Single and combined effects of gold nanoparticles and microplastics on juveniles of the marine fish *Pomatoschistus microps*, and modulation by temperature

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Mestrado em Bioquímica
Departamento de Química e Bioquímica
2014
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Acknowledgements

Reservo esta página para agradecer a todos aqueles que contribuíram para este ano tenha sido enriquecedor não só a nível académico mas também a nível pessoal.

Quero agradecer à minha orientadora Professora Doutora Lúcia Guilhermino pela orientação, disponibilidade e confiança tendo contribuído para que esta dissertação tenha sido uma experiência enriquecedora.

Agradeço também colegas de laboratório em particular ao Luís Luís pela ajuda disponibilizada sempre que necessitei e à Elsa Fonte pelo companheirismo que contribuiu para que este ano no laboratório tivesse sido gratificante.

Agradeço à minha família, aos meus pais e irmã pelo apoio durante todo o percurso.

Endereço também o meu agradecimento ao Prof. Felix Carvalho e Engª Maria Elisa Soares do laboratório de toxicologia da Faculdade de Farmácia da UP pelas determinações de ouro nos peixes e meios.

This Thesis was performed in the scope of the research projects *"SIGNAL – Effects of pollution on estuarine zooplankton-zooplanktivorous fish ecological interactions in relation to climate changes"* and *"ECORISK – Ecological risk assessment of oils and hazardous and noxious substances in the NW Portuguese coast"*, coordinated by Prof. Lúcia Guilhermino (Principal Investigator). The study was financially supported by national (Ministério da Educação e Ciência) and European funds from the European Regional Development Fund (ERDF) through the Portuguese Foundation for the Science and Technology (FCT) and the Operational Competitiveness Programme (COMPETE), under the projects "SIGNAL" (PTDC/AAC-AMB/110331/2009; FCOMP-01-0124-FEDER-013876) and Pest-C/MAR/LA0015/2013, and by the . ECORISK project (reference NORTE-07-0124-FEDER-000054), co-financed by the North Portugal Regional Operational Programme (ON.2 – O Novo Norte), under the National Strategic Reference Framework (NSRF), through the European Regional Development Fund (ERDF), and by funds of the Institute of Biomedical Sciences of Abel Salazar (ICBAS), University of Porto, attributed to the Department of Populations Study and the Laboratory of Ecotoxicology.

Resumo

Atualmente, existe uma grande preocupação em torno dos contaminantes emergentes como os microplásticos e os nanomateriais. Esta preocupação advém do facto de estes serem usados intensamente resultando na sua entrada nos ecossistemas e consequente contaminação e dos potenciais efeitos adversos que podem causar em humanos e outros organismos. Visto que os seus efeitos começaram a ser investigados apenas recentemente, o conhecimento quer do seu comportamento no ambiente quer dos efeitos nos organismos e da sua interação toxicológica é escasso. É então necessário aumentar este conhecimento para uma melhor avaliação dos riscos ambientais e dos riscos para o Homem que estes contaminantes acarretam. A temperatura é um fator que tem ganho relevância em estudos toxicológicos e ecotoxicológicos uma vez que tem sido demonstrado que esta influencia a toxicidade de vários contaminantes e vários mecanismos de defesa contra xenobióticos. Assim, considerando o cenário de aquecimento global e a elevada diversidade de contaminantes ambientais, é necessária mais investigação para responder aos desafios de gestão ambiental para enfrentar e mitigar os efeitos decorrentes das mudanças climáticas globais.

O principal objetivo do presente estudo foi investigar os efeitos tóxicos das nanopartículas de ouro (AuNP), sozinhas ou combinadas com microplásticos (MP), em juvenis do peixe estuarino *Pomatoschistus microps* e a potencial modulação do processo pela temperatura. Quatro hipóteses foram testadas: (i) os peixes expostos a AuNP absorvem ouro através da água; (ii) AuNP (5 nm de diâmetro) são tóxicas para *P. microps* juvenis em concentrações de exposição na faixa de ppb; (iii) MP (esferas de polietileno de 1-5 µm) interagem com os efeitos das AuNP em *P. microps* juvenis; e (iv) o aumento da temperatura de 20°C para 25°C influencia os efeitos destas substâncias sobre os juvenis de *P. microps*. *P. microps* foi a espécie escolhida como modelo para este estudo, principalmente porque é uma espécie chave nos ecossistemas onde ela ocorre e uma presa importante para peixes utilizados para o consumo humano. Numa primeira fase do estudo, um método espectrofotométrico e um método de fluorescência para determinar as concentrações reais das AuNP e MP em água do mar artificial (ASW) foram adaptados e validados. Depois, foi realizado um primeiro bioensaio de 96 horas (h) a 20°C para uma avaliação preliminar da toxicidade das AuNP para *P. microps* juvenis. Durante este bioensaio, as AuNP sofreram um decaimento significativo após 48 h em ASW que foi usada como meio de teste. Além disso, foi registada uma alta taxa de mortalidade em todos os tratamentos (0,5 e 1,0 $mg.L^{-1}$ de AuNP). Portanto, decidiu-se renovar o meio de teste às 48h e expor o peixe a uma concentração AuNP inferior (0.2 $mg.L^{-1}$) em testes seguintes. Por último, um bioensaio “multi-stress” foi realizado expondo

grupos de peixe em ASW apenas (controlo), AUNP (0,2 $mg.L^{-1}$), MP (0,184 $mg.L^{-1}$), e simultaneamente a AUNP (0,2 $mg.L^{-1}$) e MP (0,184 $mg.L^{-1}$) a 20°C e 25°C. O *design* experimental incluiu 3 recipientes de teste adicionais, sem peixe, por tratamento para ambas as temperaturas. O comportamento das AuNP em ASW após 48h foi também avaliado a ambas as temperaturas, utilizando concentrações mais elevadas. Estes recipientes estiveram submetidos às mesmas condições que os recipientes que continham os peixes. Não foi fornecida alimentação aos peixes durante o período de exposição (96h), os meios de teste (ASW, 18 $g.L^{-1}$ de salinidade) foram renovadas às 48h, e foram utilizados 15 peixes por cada tratamento e temperatura. No início do bioensaio, e em cada 24 horas, a mortalidade dos peixes, a temperatura da água, os níveis de oxigénio e pH foram determinados e foram recolhidas amostras para a determinação das concentrações das AuNP e de MP. Após o período de exposição, a performance predatória de todos os peixes foi avaliada. Seis peixes por tratamento e temperatura foram utilizados para a determinação do conteúdo corporal de ouro. Os restantes foram utilizados para a determinação dos seguintes biomarcadores: os níveis de peroxidação lipídica (LPO) e as atividades das enzimas acetilcolinesterase (AChE); glutationa s-transferase (GST) e etoxiresorufina-O-desetilase (EROD).

Os resultados obtidos indicam que as AuNP sofrem alterações no seu tamanho e forma ao longo do período de renovação de meios de teste (48 h) e a temperatura influencia este processo. As concentrações de AuNP e MP nos meios de teste decaíram durante este intervalo de tempo, com efeitos superiores a 25°C (40-57% e 27-46% de decaimento para AuNP e MP respetivamente) do que a 20°C (36-49% e 25-38% de decaimento para AuNP e MP respetivamente), provavelmente devido à ligação entre partículas, a agregação e precipitação. Além disso, o decaimento foi maior na presença de peixes (30-57% e 23-46% para AuNP e MP, respetivamente) do que na sua ausência (36-46% e 25-41% para AuNP e MP respetivamente), sugerindo que ocorreu absorção destas partículas pelos peixes e/ou, os seus produtos metabólicos interagiram com as partículas. Estes resultados ilustram algumas das dificuldades dos testes de toxicidade com estes contaminantes ambientais, especialmente em água salgada.

Os peixes expostos às AuNP isoladamente e em combinação com o MP continham concentrações médias de ouro nos seus corpos de 0,24 e 0,13 $\mu g.g^{-1}$ a 20°C e de 1,07 e 0,55 $\mu g.g^{-1}$ a 25°C. Estes resultados indicam que sob exposição a AuNP os peixes captam ouro da água, tal como sugeriam os dados do decaimento das AuNP, corroborando assim a nossa primeira hipótese. Os peixes expostos a AuNP e MP em conjunto continham menos ouro nos seus corpos do que quando expostos a apenas AuNP; além disso, o decaimento das AuNP foi mais elevado na presença de peixes do

que na sua ausência, e as concentrações de ouro em água não eram significativamente diferentes nos tratamentos testados. Estes resultados indicam que, além de interações diretas entre MP e AuNP no meio de teste, os MP interagem com o *uptake* das AuNP. A exposição a AuNP (0,2 $mg.L^{-1}$), quer como substancia única ou combinada com MP, diminuiu significativamente o desempenho predatório dos peixes, um efeito que pode conduzir a uma diminuição da performance individual no ambiente e pode conduzir à morte tendo efeitos negativos sobre a taxa de crescimento da população. Estes resultados indicam que as AuNP são tóxicas para os peixes na faixa de ppb (segunda hipótese). MP sozinhos também são tóxicos para os peixes visto que induziram dano oxidativo, como indicado pelo aumento dos níveis de LPO. Não foram detetados efeitos significativos sobre os outros biomarcadores. Como discutido acima, os dados de decaimento das AuNP e dos MP sugerem que há interações diretas entre as duas substâncias no meio de teste, e foram encontradas concentrações mais elevada de ouro no corpo dos peixes expostos a AuNP do que nos expostos simultaneamente às duas substâncias testadas. Além disso, foram detetados menores (não significativamente diferentes do grupo controlo) níveis de LPO nos peixes expostos simultaneamente a AuNP e MP do que naqueles expostos apenas aos MP, sugerindo que as AuNP podem prevenir os danos da oxidação lipídica induzidos pelos MP. Estes resultados corroboram a nossa terceira hipótese. Finalmente, para além da influência da temperatura sobre o decaimento das substancias no meio de teste, a concentração de ouro no corpo dos peixes foi cerca de 2 vezes superiores a 25°C do que a 20ºC. Além disso, houve uma redução na atividade da GST em peixes expostos a AuNP e a MP separadamente a 25°C relativamente aos expostos aos mesmos tratamentos a 20°C, um efeito que é provável que reduza a capacidade dos peixes de responder ao *stress* induzido por xenobióticos, observou-se também um aumento da atividade da EROD em peixes expostos a 25°C em relação aos expostos a 20ºC. Estes resultados indicam uma influência da temperatura sobre a taxa de decaimento de MP e AuNP no meio de teste e, portanto, nas concentrações de exposição, no *uptake* de ouro por peixes, nos efeitos tóxicos e nalgumas respostas dos peixes ao *stress* químico, corroborando a nossa quarta hipótese.

Em resumo, tanto as AuNP como os MP, que são contaminantes emergentes, induziram efeitos tóxicos sobre *P. microps* juvenis que podem diminuir a sua sobrevivência e desempenho na natureza. Ocorreram interações toxicológicas entre AuNP e MP em *P. microps* juvenis e a temperatura influenciou o comportamento e os efeitos tóxicos destas substâncias.

Palavras-chave: Contaminantes emergentes; nanopartículas de ouro; microplásticos; misturas; temperatura; *Pomatoschistus microps;* performance predatória; biomarcadores

Abstract

Currently there is a high concern about emerging contaminants such as microplastics and nanomaterials. This concern comes from the widely and intensive use of these substances, the consequent environmental contamination resulting from their entrance into the ecosystems, and the adverse effects that they may cause on humans and the wildlife. Since their toxic effects started to be investigated only recently, the knowledge on their fate both inside the organisms and in the environment, and on their effects on wild organisms is limited, and even more scarce is the knowledge on the potential toxicological interactions between microplastics and nanoparticles. This knowledge is most important to improve the basis for environmental and human risk assessment of these substances. Temperature is a factor that has gained relevance in toxicological and ecotoxicological studies since it has been shown that temperature influences the toxicity of some common environmental contaminants and several mechanisms of defence against the toxic insult in several species, including humans. Thus, considering the global warming scenario and the high diversity of environmental contaminants, more research is needed to respond the demands of environmental management to face and mitigate the effects resulting from global climate changes.

The main goal of the present study was to investigate the toxic effects of gold nanoparticles (AuNP), alone and combined with microplastics (MP), on juveniles of the estuarine fish *Pomatoschistus microps* and the potential modulation of the process by temperature. Four hypotheses were tested: (i) fish exposed to AuNP through the water uptake gold; (ii) AuNP (5 nm diameter) are toxic to *P. microps* juveniles at exposure concentrations in the ppb range; (iii) MP (polyethylene 1-5 µm spheres) interact with the effects of AuNP on *P. microps* juveniles; and (iv) the rise of temperature from 20ºC to 25ºC influences the effects of chemicals on *P. microps* juveniles. *P. microps* was selected as model for this study mainly because is a key-stone species in the ecosystems where it occurs and an important prey for fish used for human consumption. In a first phase of the study, a spectrophotometric method and a spectrofluorimetric method to determine the actual concentrations of AuNP and MP in artificial sea water (ASW) were adapted and validated. Then, a first 96 hours (h) toxicity bioassay was carried out at 20ºC to preliminary assess the toxicity of AuNP to *P. microps* juveniles with previously acclimatized fish. During this bioassay an important decay of the tested substance was found after 48 h in ASW that was used as test medium. In addition, a high mortality was recorded in all treatments, including 0.5 and 1.0 $mg.L^{-1}$ of AuNP. Therefore, it was decided to change test media at 48h, and to expose the fish to a lower AuNP concentration (0.2

mg.L^{-1}) in further toxicity tests. Finally, a multi-stressors bioassay was performed by exposing groups of fish to ASW only (control), AuNP (0.2 mg.L^{-1}), MP (0.184 mg.L^{-1}), and simultaneously to AuNP (0.2 mg.L^{-1}) and MP (0.184 mg.L^{-1}) at 20ºC and 25ºC. The exposure design included 3 additional test beakers without fish per treatment at both temperatures. The behaviour of the AuNP in ASW in 48h was also assessed at both temperatures using higher AuNP concentrations. These test beakers were submitted to the same conditions of those having fish. No food was provided to the fish during the exposure period (96h), the test media (ASW, 18 g.L^{-1} salinity) were renewed at 48h, and 15 fish were used per treatment and temperature. At the beginning of the bioassay and at each 24h, fish mortality was recorded, water temperature, oxygen and pH were determined, and samples for determination of AuNP and MP concentrations were collected. After the exposure period, the post-exposure predatory performance (hereafter indicated as predatory performance) of all the fish was assessed. Six fish per treatment and temperature were used for determination of their gold body content. The remaining ones were used for determination of the following biomarkers: the levels of lipid peroxidation (LPO) and the activities of the enzymes acetylcholinesterase (AChE); glutathione s-transferases (GST) and ethoxyresorufin-O-deethylase (EROD).

The results obtained indicate that AuNP change their size and shape along the test media renewal period (48h) and temperature influences the process. The concentrations of AuNP and MP in test media decayed during this time interval, with higher effects at 25ºC (40-57% decay 27-46% for AuNP and MP, respectively) than at 20ºC (36-49 % decay 25-38% for AuNP and MP, respectively), likely due to binding, aggregation and precipitation. Moreover, the decay was higher in the presence of fish (30-57% and 23-46% for AuNP and MP, respectively) than in their absence (36-46% and 25-41% for AuNP and MP, respectively) suggesting that fish uptake both chemicals from the water and/or their metabolic products interact with the particles. These results illustrate some of the difficulties of toxicity testing with these environmental contaminants, especially in saltwater.

Fish exposed to AuNP alone and in combination with MP had mean concentrations of gold in their body of 0.24 and 0.13 µg.g^{-1} at 20ºC and of 1.07 and 0.55 µg.g^{-1} at 25ºC. These findings indicate that under exposure to AuNP fish uptake gold from the water as suggested by AuNP decay data, thus corroborating our first hypothesis. When simultaneously exposed to AuNP and MP, fish had less gold in their bodies than when exposed to AuNP alone; in addition, the AuNP decay was higher in the presence of fish than its absence, and the gold concentration in the water were not significantly different at the treatments tested. Taken together, these results indicate that in addition to direct

interactions between MP and AuNP in test media, toxicological interactions during AuNP uptake occur. The concentration of AuNP tested (0.2 $mg.L^{-1}$), either as single substance or combined with MP, significantly decreased the predatory performance of fish, an effect that is expected to result in a decrease of the individual fitness and may lead to death with potential negative effects on the population growth rate. These results indicate that AuNP are toxic to fish in the ppb range (second hypothesis). MP alone are also toxic to fish inducing lipid oxidative damage as indicated by the rise of LPO levels. No significant effects on the other biomarkers were found. As discussed above, AuNP and MP decay data suggest direct interactions between the two substances in test media, and a higher concentration of gold was found in the body of fish exposed to AuNP alone than in those exposed simultaneously to the two tested substances. Furthermore, lower (and not significantly different from the control group) LPO levels were found in fish exposed simultaneously to AuNP and MP than in those exposed to MP alone, suggesting that AuNP prevent the lipid oxidation damage induced by MP. These results corroborate our third hypothesis. Finally, in addition to the influence of temperature on the decay of test substances in test media, the concentration of gold in fish body was about 2 folds higher at 25ºC than at 20ºC. In addition, a reduction of GST activity in fish exposed to AuNP and to MP separately at 25ºC relatively to those exposed to the same treatments at 20ºC, an effect that is likely to reduce the ability of fish to respond to chemically induced stress, and an increase of EROD activity was observed in fish exposed to AuNP and MP at 25ºC but not at 20ºC. These results indicate an influence of temperature on the rate of AuNP and MP decay in test media and thus in the exposure concentrations, in the uptake of gold by fish, on the toxic effects and in some important responses of fish to chemical stress, corroborating our fourth hypothesis.

In summary, both AuNP and MP that are emerging contaminants of high concern induced toxic effects on *P. microps* juveniles that may decrease their survival and performance in the wild. Toxicological interactions between AuNP and MP in *P. microps* juveniles occurred and temperature influence the fate and toxic effects of the chemicals.

Keywords: Emerging contaminants; gold nanoparticles; microplastics; mixtures; temperature; *Pomatoschistus microps*; predatory performance; biomarkers

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List of Abbreviations

ACh – Acetylcholine

AChE – Acetylcholinesterase

AgNP – Silver Nanoparticles

ANOVA – Analysis of Variance

ASW – Artificial Sea Water

AuNP – Gold Nanoparticles

BCF – Bioconcentration Factor

CDNB - 1-chloro-2,4-dinitrobenzene

ChE – Cholinesterase

CYP – Cytochrome P450

CYP1A – Cytochrome P450 subfamily 1A

DTNB – 5,5'-dithiobis-(2-nitrobenzoic acid)

EROD - Ethoxyresorufin-O-deethylase

F - Fluorescence

GSH – Glutathione

GST – Glutathione S-Transferases

IUPAC - International Union of Pure and Applied Chemistry

LC_{50} – Median Lethal Concentration: the estimated concentration needed to cause the mortality of 50% of the studied population in the specific conditions of the toxicity test

LPO – Lipid Peroxidation

MSFD – Marine Strategy Framework Directive

MP – Microplastics

OD – Optical Density

OECD - Organisation for Economic Co-operation and Development

PAHs – Polycyclic Aromatic Hydrocarbons

ROS – Reactive Oxygen Species

SOD – Superoxide Dismutase

TBA - Thiobarbituric Acid

TBARS – Thiobarbituric Acid Reactive Substances

TCA – Trichloroacetic Acid

TNB - 5-thio-2-nitrobenzoic acid

u.p. – Ultra Pure

1. Introduction

Environmental pollution and aquatic in particular, has been a problem since ancient times (Islam & Tanaka, 2004) contributing in the past to severe epidemics that caused a significant number of human casualties (Novotny, 2003). Today, pollution still is a major source of human health risk in the world, with more severe situations in developing countries (Briggs, 2003). Healthy ecosystems are fundamental for the human society, providing most valuable services, known as "ecosystem services", namely provisioning (e.g. water, food, materials), regulating (e.g. climate regulation, carbon sequestration, disease control), and cultural (e.g. recreational, educational, spiritual, symbolic) ones (MEA, 2005). The ecosystem services concept provides a useful link between natural resources and social benefits, making more visible the crucial importance of environmental quality to human health and development (Pinto, et al., 2014).

The pollution of the marine environment has been generating increasing concerns due to the negative impacts that it can have on human health (e.g. through the ingestion of contaminated fish, or by performing recreational activities in polluted areas) and on marine ecosystem services (e.g. fishery decrease, reduction of water quality and/or quantity). Among marine ecosystems, estuaries and other coastal ecosystems located in densely populated and/or industrialized areas are at particular risk because they are under multi-stressors exposure as the result of anthropogenic activities (e.g. agriculture in neighbour fields, harbour activity, offshore oil extraction) (Basset, et al., 2013; Gonçalves, et al., 2013; Guimarães, et al., 2012) and natural processes such as storms, floods and droughts (Jennerjahn & Mitchell, 2013). These stressors often include water and sediment contamination by a high number of different chemical substances; habitat degradation and loss; alterations due to climate changes; invasions by exotic invasive species, including pathogens, among several others (Gamito, 2008; Jennerjahn & Mitchell, 2013). The combined effects of these stressors on environmental and human health are largely unknown and more knowledge is urgently needed, as indicated in several regulations like the Marine Strategy Framework Directive (MSFD) (European Commission, 2012) and research programs such as the Horizon 2020.

The environmental contaminants present in estuaries and other coastal areas include substances known for long time, which are often designed by "classical" or "traditional" environmental contaminants, such as metals, persistent organic pollutants (POPs), detergents, among several others. They also include substances identified as environmental contaminants only recently, known as "emerging environmental

contaminants of high concern" or just "emerging contaminants" such as pharmaceuticals (Thomaidis, et al., 2012), nanomaterials (Boxall, 2012), and microplastics (Browne, et al., 2007). Because these substances are widely and intensively used, they have been found in the environment and in the organisms of several species, including in species used for human consumption (Calderón-Preciado, et al., 2011; Gil, et al., 2012; Meredith-Williams, et al., 2012; Setälä, et al., 2014; Stewart, et al., 2014). The knowledge on the effects of emerging contaminants on wild organisms and ecosystems (and in some cases also in human health) is scarce, and more knowledge is urgently needed (Martins, et al., 2013; Oliveira, et al., 2013; Thomaidis, et al., 2012)

Global warming is considered a major environmental thread with very concerning potential effects on human and environmental health. In estuaries and other coastal areas, it is expected to have major impacts with effects already visible in several regions (Isobe, 2013; Wernberg, et al., 2011). Among other effects, important variations of water temperature are expected to occur in lakes, estuaries and other coastal areas (Almeida, et al., 2014; Mooij, et al., 2008) that will be a challenge to the survival and performance of several species. Temperature variation influence a wide range of biological and ecological processes (Almeida, et al., 2014; Segner, et al., 2014) and it can be a major environmental stressor. In recent studies, it has been found to interact with the toxicity of several contaminants like metals (Zeng & Wang, 2011), pesticides (Laetz, et al., 2014) and pharmaceuticals (Martins, et al., 2013) a most important finding also in relation to human health. However, the knowledge on the potential impacts resulting from temperature-pollutants interactions is still limited. Improving this knowledge is a priority because humans and wildlife are exposed to such effects.

1.1. Microplastics

Due to their versatility, plastics have been used in increasingly diverse products of industrial, medical and domestic application that are used globally in an intensive way, consequently, their production has been rising reaching in 2012 a worldwide production up to 288 million tones (PlasticsEurope, 2013). Mainly due to indiscriminate disposal a significant part of the plastic produced end up directly or indirectly in the marine environment where they may persist and accumulate (Cole, et al., 2011). In European Union countries half of all plastic waste is recovered for further utilization (OECD, 2010). However, globally, the amount of plastic debris found in environmental compartments is still growing (Oliveira, et al., 2013).

In the environment, plastics are fragmented into smaller particles in a progressive and very slow process until reaching the micro or lower scale (Browne, et al., 2007). Such plastic debris are known as secondary microplastics (MP), to distinguish them from primary MP that are specifically produced to have a microscopic size (e.g. microspheres used as industrial abrasives, cosmetics and other consumer products) (Arthur, et al., 2009). Recently, the United States of America (U.S.A.) National Oceanic and Atmospheric Administration (NOAA) defined MP as plastic particles smaller than 5 mm.

In the marine environment, where MP have been particularly studied, they have been widely found, including in remote regions, such as the Arctic (Ivar do Sul & Costa, 2014). The highest concentrations were found in oceanic gyres and in estuaries and other coastal areas of densely populated and heavily industrialized regions (Wright, et al., 2013). For example, concentrations up to 197 particles (size < 5mm) per m^3 and up to 1 particle per $25cm^3$ were found in sediments (sand) in Portuguese beaches (Martins & Sobral, 2011) and in deep sea sediments on the Atlantic Ocean (Van Cauwenberghe, et al., 2013) respectively; and concentrations up to 9800 particles.m^{-3} and 779 particles.L^{-1} were found in water samples from the northeastern Pacific Ocean (Desforges, et al., 2014) and in Norwegian coastal waters respectively (Norén & Naustvoll, 2010). Fibrous microplastics are the most abundant in the marine environment (Wright, et al., 2013). Several plastic classes are used on the production of plastic products like polyethyelene, polypropylene, polystyrene, poly(ethylene terephthalate); and poly(vinyl chloride) being polypropylene the most produced (PlasticsEurope, 2013) and the most encountered on marine environment (Andrady, 2011).

Marine organisms are able to uptake MP from the environment, including planktonic organisms such as zooplankton, larval fish, copepods and salps (von Moos, et al., 2012; Hollman, et al., 2013), fish (Boerger, et al., 2010; Lusher, et al., 2013), birds (van Franeker, et al., 2011) and probably whales (Fossi, et al., 2012). Several animals, including fish, ingest plastic particles by confusion with food (Boerger, et al., 2010; Moore, 2008) particularly if their color resemble the fish natural prey (Wright, et al., 2013). The ingestion of MP by fish was reported in diverse regions of the globe. On the English Channel, ten different species of fish were found to have ingested plastic after examination of their gastrointestinal tracts. The majority of the ingested plastic consisted of fibers and fragments. Microplastics corresponded to 92.4% of the total ingested plastic (Lusher, et al., 2013). On the North Sea plastic particles were found on five different species. The particles found had a size lower than 5mm. The particles found were of polyethylene, polypropylene, polyethylene terephthalate and styrene-acrylate (Foekema,

et al., 2013). Planktivorous fish of the north Pacific central gyre were found to ingest plastic particles consisting primarily of fragments. The ingestion of plastic was found to increase with fish size (Boerger, et al., 2010). Recently, it has been found that MP can be transferred from the base to the top of food chain (Setälä, et al., 2014) which can be a matter of concern to human health due to the importance of fish in human diet.

Impacts of ingested MP include internal and/or external abrasions and ulcers; blockages of the digestive tract, which can result in false satiation, starvation and physical deterioration among other effects that may diminish reproduction, the ability to escape from predators, feeding capacity potentially leading to dead (Wright, et al., 2013). Beyond the physical harm MP can cause if ingested, other toxic effects are possible. Oliveira *et al.* (2013) reported significant inhibition of Acetylcholinesterase (AChE) activity in *Pomatoschistus microps* by MP and, in the same work, it was observed that microplastics exacerbate pyrene toxicity on AChE and Isocitrate dehydrogenase (IDH) (pyrene and microplastics alone didn't inhibit IDH) suggesting a toxicological interaction of microplastics with polycyclic aromatic hydrocarbons (PAHs). Additionally, MP may contain chemicals introduced during their manufacture and, due to the large surface area to volume ratio of MP, aqueous metals and organic pollutants present on the environment are adsorbed and concentrate on their hydrophobic surface (Cole, et al., 2011; Rios, et al., 2007). Ingestion of MP with adhered pollutants introduces hazardous contaminants to the organisms and may be transferred along trophic chains (Setälä, et al., 2014), so, top predators like humans may be exposed to these chemicals.

In summary, because MP can induce adverse effects on wild organisms and humans and are widely and intensively used, they are considered environmental contaminants of particular concern, their environmental concentrations should be monitored, including in the scope of the Marine Strategy Framework Directive (MSFD), and their effects on organisms should be further investigated (Galgani, et al., 2013; Wright, et al., 2013), especially regarding the interactions with other environmental contaminants of high concern and their potential adverse effects to top predators, including humans.

1.2. Nanomaterials

Manufactured nanomaterials are man-made structures, ranging in size from 1 nanometer (nm) to 100nm (Motzer, 2008). Thanks to their unique properties, nanomaterials are now applied in a wide variety of everyday products like cosmetics, sporting products, tires, stain-resistant clothing, sunscreens, toothpaste and food additives (Buzea, et al., 2007). They are also widely used in other applications such as medicine

(e.g. drug delivery, biosensors), environmental technologies (e.g. nanofiltration) and information and communication technologies (e.g. nanoelectronic and optoelectronic materials, organic light emitters) (Motzer, 2008). There are different classes of manufactured nanomaterials, including: carbon based materials such as nanotubes; metal based materials such as metal oxides nanoparticles, gold and silver nanoparticles; dendrimers which are spherical polymeric molecules constructed from branched units; bioinorganic composites which are hybrid nanomaterials composed by an inorganic material and biological material, for instance, of gold nanoparticles and chitosan.

The expansion of the nanotechnology field and the widespread of nanomaterials across a vast variety of products contribute to the introduction of considerable amounts of these substances in the environment (Rana & Kalaichelvan, 2013). Manufactured nanomaterials enter the environment through wastewaters and solid waste from domestic and industrial activity and atmospheric emissions (Lapresta-Fernández, et al., 2012). In respect to the aquatic environment, manufactured nanoparticles travel from surface, ground and underground water, until they reach estuaries and oceans passing through different conditions of salinity, pH and ionic strength (Lapresta-Fernández, et al., 2012) which can alter their properties and ultimately their toxicity. Since nanomaterials are more likely to accumulate than to disappear from the ecosystems (Rana & Kalaichelvan, 2013), it is important to gather more knowledge about their potential effects on biota. In addition to these man-made nanomaterials there are naturally occurring nanoparticles. For example, natural phenomena like dust storms and volcanic eruptions are major source of nanoparticles in the environment (Buzea, et al., 2007).

At nanoscale, the surface chemistry changes due to the larger surface-to-volume ratio which increases the fraction of atoms at the surface of nanoparticles when compared to larger particles (Hosokawa, et al., 2007), increasing their reactivity (Elsaesser & Howard, 2012). Thus, depending on the dose, size, form, and other properties, the toxicological effects of a specific nanomaterial might be considerably different from the corresponding macro and micro sized materials. In general, the nanoscopic dimensions of nanomaterials allow them to be rapidly absorbed by organisms, translocate into the circulatory or lymphatic systems (Buzea, et al., 2007), to cross de blood-brain barrier (if smaller than 12nm) and to be endocytosed by cells (for particles with less than 30nm in diameter) (Alkilany & Murphy, 2010) either via active or passive mechanisms (Elsaesser & Howard, 2012). These properties are very interesting for biomedical applications like medical imaging or drug delivery but they are as well an eventual source of toxicity, for example, their entrance into cells can produce oxidative and organelle damage (Buzea, et al.,

2007). The nanotoxicology is a recent field, with several difficulties mainly because the available methods often are not adequate to assess the toxicity of nanoparticles. Therefore, the knowledge is still limited despite the considerable work that has been done in the last years, especially in relation to wild species and ecosystems, and even more in marine ones.

Among nanoparticles, gold nanoparticles (AuNP) are of special interest mainly because they have most important applications in medicine and biomedicine. For example, they can be used as photo thermal therapy agent and imaging agents (Khan, et al., 2013) on drug delivery (Pissuwan, et al., 2011) and on other biomedical and chemical applications (Khan, et al., 2013). As for other nanoparticles, their biodistribution and toxic effects largely depend on the size, coating, among other factors (Alkilany & Murphy, 2010; Cho, et al., 2009; Fraga, et al., 2014; Sonavane, et al., 2008). When testing aquatic species, the composition of the test medium is a factor of crucial importance because its components may interact with the AuNP, changing their bioavailability. In bioassays with marine species, this is even more complicated because saltwater needs to be used.

In mammals where AuNP have been most studied, and depending on the properties affecting their effects, they were found to accumulate in organs (De Jong, et al., 2008; Fraga, et al., 2014), to cross the blood-brain barrier (De Jong, et al., 2008; Lasagna-Reeves, et al., 2010; Sonavane, et al., 2008), to induce inflammation and apoptosis in liver tissues in mice (Cho, et al., 2009), severe sickness and lethality in mice exposed to particles between 8-37nm (Chen, et al., 2009), DNA damage in the cerebral cortex of adult rats (Cardoso, et al., 2014) and in some works no obvious toxic effects were detected (Fraga, et al., 2014; Lasagna-Reeves, et al., 2010). There are also some toxicity studies in aquatic species that are summarized in Table 1.

Table 1 – Toxicity of gold nanoparticles in aquatic organisms. AuNP – gold nanoparticles; AgNP – silver nanoparticles: DOC - dissolved organic carbon. Ref. - Reference

Material (Size)	Concentration	Exposure	Organisms	Environment	Effects Observed	Ref.
AuNPs (5.3 ± 1nm)	750 ppb	24h	*Mytilus edulis*	Salt water; Salinity: 34-36 ‰ ; 15-16ºC	Decreased amount of thiol-containing proteins; Lipid peroxidation in digestive gland; decreased lysosomal membrane stability.	(Tedesco, et al., 2010)
AuNPs (5-10nm) AgNPs (1-10nm) AuNPs with DOC AgNPs with DOC	0.058 to 17.4 $mg.L^{-1}$ 0.063 to 19 $mg.L^{-1}$ 0.058 to 17.4 $mg.L^{-1}$ 0.063 to 19 $mg.L^{-1}$	48h	*Oncorhynchus mykiss* (hepatocytes)	Cell Culture Leibowitz 15 for cytotoxic assays and Krebs ringer-HEPES buffer for ROS	**AuNPs**: Induction of ROS formation; no cytotoxicity. **AgNPs**: Highly cytotoxic (reduction of metabolic activity and membrane integrity); EC50 between 2.5 $mg.L^{-1}$ and 4.9 $mg.L^{-1}$; No ROS formation. Similar effects with NPs with DOC.	(Farkas, et al., 2010)
AuNPs (5; 15 and 40 nm)	100 $\mu g.L^{-1}$	16d	*Scrobicularia plana*	Pre-filtered natural seawater (0.45 µm); 10ºC	Bioaccumulation in soft tissues. **Increased** metallothionein for 5 and 40nm AuNPs; Catalase for 15 and 40nm; GST for all three sizes (no differences between sizes); SOD for 40nm and AChE for 15 and 40nm. Decreased burrowing kinetics after 7d of pre-exposure (stronger effect with increasing size).	(Pan, et al., 2012)
Non functionalized AuNPs and hyaluronic acid capped AuNPs (12.5nm)	10$mg.L^{-1}$ (algae test) 20$mg.L^{-1}$ (fish test) 100$mg.L^{-1}$ (f *Daphnia* test)	96h	*Scenedesmus subspicatus*; *Daphnia magna; Brachydanio rerio*	Freshwater	Non capped AuNPs distributed around algae cell wall but didn't penetrate it. Both types of AuNPs found on digestive tract of *Daphnia* and fish. No mortality or overt toxicity.	(García-Cambero, et al., 2013)

Several of these studies indicate that AuNP are able to enter into aquatic species, probably by several routes, and accumulate in their tissues. The comparison of different studies also suggests that the test medium, the model organism, and other factors, including the type of exposure and the range of concentrations tested, influence the results, in addition to the properties of the AuNP tested. In the marine bivalve *Scorbicularia plana* exposed to AuNPs (5; 15 and 40 nm) water concentrations of 100 $\mu g.L^{-1}$ for 16 days, an induction of the activity of the anti-oxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione S-tranferases (GST) was found indicating oxidative stress; induction of acetylcholinesterase (AChE) activity, the enzyme that hydrolyzes the neurotransmitter acetylcholine (ACh) in cholinergic synapses of vertebrates and invertebrates, was also found indicating the ability of these particles to act on the nervous system; increased metallothionein levels were also observed indicating that these proteins are involved in the response to AuNP induced stress (Pan, et al., 2012). The oberved effects were size-dependent. Oxidative stress induced by AuNP (5.3 ± 1nm) was also observed in the blue mussel (*Mytilus edulis*), exposed for 24h to a concentration of 750 ppb (Tedesco, et al., 2010). Formation of ROS was detected in rainbow trout (*Oncorhynchus mykiss*) hepatocytes after exposure to AuNP (5-10nm) uncoated and coated with dissolved organic carbon (DOC). Farkas et al. (2010) work showed also that silver nanoparticles cause more severe toxicity than AuNP, which is in agreement with previous studies (Lapresta-Fernández, et al., 2012), but no differences were detected between uncapped nanoparticles and nanoparticles capped with DOC. In the algae *Scenedesmus subspicatus*, in *Daphnia magna* and in zebrafish (*Brachydanio rerio*) exposed to 10 $mg.L^{-1}$ (algae test), 100 $mg.L^{-1}$ (Daphnia test) and 20 $mg.L^{-1}$ (fish test) of AuNP non caped or caped with hyaluronic acid for 96h no overt toxicity was detected (García-Cambero, et al., 2013).

A particular important topic where more knowledge is urgently needed is the potential interactions between nanoparticles and other environmental contaminants of high concern, especially emerging ones such as MP, because the exposure of both wild organisms and humans to a single substance is a rare event, the most common situation being the simultaneous exposure to several environmental contaminants and other stressors (Boxall, 2012).

1.3. Fish toxicity bioassays and effect criteria

Fish bioassays are used for a wide range of purposes. In Ecotoxicology, they are mainly used to assess the toxic effects of chemicals and other stressors on secondary or higher predators, being required by national and European regulations in the scope of the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH).

Fish toxicity bioassays may be carried out in real scenarios (e.g. *in situ* bioassays) or in laboratory conditions with wild species or with standard species produced and maintained permanently in laboratorial conditions (Merulla & van der Meer, 2010). The type of bioassay and the test species should be selected primary according the specific objectives of the study. Either with wild specimens or standard ones, laboratory bioassays should follow international protocols (e.g. OECD 1992), assuring that the environmental conditions provided are adequate, the 3 R's principles of animal experimentation are fulfilled, and the methods are suitable to obtain results of high quality. The laboratorial bioassays with juvenile and adult fish may be acute, when testing a relatively high concentration of the test substance for a period relatively short considering the life-cycle of the species (generally 96h), chronic when testing a relatively low concentration of the test substance for a considerable period of time (e.g. several weeks, months of years), or of other types if testing cells, embryos or other biological material (Landis & Yu, 2004). The required effect criteria in standard toxicity bioassays with juvenile and adult fish are mortality, growth and reproduction. However, several other parameters have been intensively used in the last decades, including behavioural parameters and sub-individual endpoints, often called environmental biomarkers or biomarkers only (van der Oost, et al., 2003).

1.4. Environmental biomarkers

In environmental studies aimed at assessing the effects of stressors on the biota, biomarkers have been used as parameters measured in the biota at individual or sub-individual levels of biological organization (Walker, et al., 2006). According the International Union of Pure and Applied Chemistry (IUPAC), "biomarker is a quantifiable behavioural, physiological, histological, biochemical, or genetic property that is used to measure a response to an environmental change" (IUPAC, 2009). This definition will be used in the present study.

In Ecotoxicology, biomarkers can be used to assess the quality of aquatic ecosystems since they can reflect the exposure and/or effects of organisms, populations and communities to environmental contaminants and other stressors (e.g. temperature)

(van der Oost, et al., 2003; Walker, et al., 2006). Since the interactions of stressors with the organisms occur first at molecular level being reflected at successively higher organization levels, if the protection and repair mechanisms at each level are overcome, biomarkers can be most valuable early indicators of adverse effects (Klaassen, 2008; van der Oost, et al., 2003). Biomarkers can be used in field studies, such as monitoring studies in wild populations (Gravato, et al., 2008) and in *in situ* bioassays (Castro, et al., 2004), and as effect criteria in laboratory bioassays (e.g. Oliveira et al. 2013; Vieira et al. 2008; Vieira et al. 2009) as previously indicated (section 1.3). Enzymes involved in functions crucial for the survival and fitness of organisms (e.g. neuro system functioning, xenobiotic biotransformation, oxidative stress defenses), and parameters indicative of biological damage (e.g. lipid peroxidation) have been widely and intensively used as biomarkers in environmental studies for decades (van der Oost, et al., 2003). More recently, behavioural parameters have been increasingly used because they provide a most important link between effects at sub-individual levels and those observed at individual level, and in some cases, directly related with negative effects on the population fitness (Hellou, 2011).

The biotransformation of xenobiotics is a series of enzyme catalyzed reactions that intend to alter the physicochemical properties of the xenobiotics in order to make them more hydrophilic, favoring their excretion through urine and bile and, consequently, reducing their lipophilicity to impair their absorption across biological membranes (Klaassen, 2008). This process is generally divided in two or three phases. In phase I, the xenobiotic is altered through oxidation (catalyzed by numerous enzymes such as xanthine dehydrogenase/oxidase, cytochrome P450 superfamily or aldehyde oxidase), hydrolysis (catalyzed mainly by carboxylesterases, cholinesterases, and paraoxonases) or reduction reactions (which can be non enzymatic or catalyzed by enzymes such as alcohol dehydrogenase, aldehyde oxidase, and cytochrome P450), the metabolite formed can then be conjugated on phase II (e.g. glucuronidation, sulfonation, methylation, acetylation, amino acid conjugation, glutathione conjugation) and catabolyzed on phase III (Klaassen, 2008; van der Oost, et al., 2003). Several of the xenobiotic biotransformation enzymes are inducible, so, when there are high levels of xenobiotics (or toxic byproducts) the enzymes involved in their detoxification will be upregulated (Klaassen, 2008). However, the activity of some of these enzymes may also be inhibited because the xenobiotic and/or it(s) metabolites bind to the enzyme with the resulting complex being inactive (Klaassen, 2008). In environmental studies, the activity of the enzymes ethoxyresorufin-O-deethylase (EROD) and glutathione S-transferases (GST) have been particularly used as indicative of

alterations induced in phase I and phase II reactions, respectively (van der Oost, et al., 2003). The cytochrome P450 (CYP) superfamily of monooxygenases intervenes on the phase I of the biotransformation process of xenobiotics catalyzing oxidation reactions. These enzymes are heme containing proteins and in vertebrates they are mainly found in liver microsomes although they are also present in several other tissues (Ogu & Maxa, 2000). The induction of CYP1A subfamily, which is the subfamily responsible for the biotransformation of numerous xenobiotics (van der Oost, et al., 2003), has been used as biomarker relatively to several common organic contaminants like polycyclic aromatic hydrocarbons (PAHs) and planar halogenated hydrocarbons (PHHs) (Whyte, et al., 2000). The evaluation of EROD activity is considered the most sensitive method to assess the induction (or inhibition) of CYP superfamily (CYP1A subfamily specifically) in response to xenobiotics in fish (van der Oost, et al., 2003). Contaminants affecting CYP function can impair phase I biotransformation, for instance, the inhibition of CYP by one toxicant can exacerbate the toxicity of a second compound due to impaired biotransformation (Klaassen, 2008). Some environmental contaminants, such as some organophosphate insecticides are able to inhibit EROD activity (Flammarion, et al., 1998). GST are a family of dimeric isoenzymes involved on the phase II of the biotransformation of xenobiotics and endogenous compounds generated during metabolic processes). In vertebrates, the concentration of these enzymes is particularly high in the liver but they are also present in several other organs (Sherratt & Hayes, 2001). They catalyze the nucleophilic attack of the reduced form of glutathione to an electrophilic carbon, sulphur or nitrogen atom of xenobiotics or phase I metabolites, preventing the interaction of those electrophilic species with other proteins and nucleic acids potentially resulting in their damage (Klaassen, 2008). However, the glutathione conjugate formed is sometimes more reactive than the parent compound in a phenomenon called bioactivation that happens, for instance, with short-chain alkyl halides that contain two functional groups (Hayes, et al., 2005). Additionally, GST are protective agents against byproducts of oxidative stress, have a key role on the synthesis of eicosanoids and steroid hormones and intervene on cell signaling pathways (Hayes, et al., 2005).

Other enzymes widely used as biomarkers are cholinesterases (ChE), a family of enzymes present in vertebrates and invertebrates (Nunes, 2011). This family includes acetylcholinesterase (AChE), known as true cholinesterase, and butyrylcholinesterase or propionylcholinesterase, also known as pseudo-cholinesterase (van der Oost, et al., 2003). AChE has a determinant function in the nervous system function because it hydrolyses the neurotransmitter acetylcholine in cholinergic synapses (Dvir, et al., 2010).

Inhibition of AChE leads to the accumulation of acetylcholine in the synaptic cleft resulting in overstimulation of the cholinergic receptors of post synaptic cells disrupting the nerve function, a process that may lead to death (Gupta, 2006). Nonetheless, AChE inhibitors are used in treatment of a variety of neurologic disorders such as Alzheimer's disease (Dvir, et al., 2010). The inhibition of ChE is frequently used as biomarker of organophosphates and carbamates of exposure and effect because these compounds strongly inhibit their activity at low concentrations (van der Oost, et al., 2003). However, ChE of several organisms are also inhibited by several other environmental contaminants, like metals, detergents and mixtures of pollutants (Guilhermino, et al., 1998; Guilhermino, et al., 2000; Payne, et al., 1996). Therefore, at the present, ChE have a more general use in environmental studies.

Lipid peroxidation (LPO), the oxidation of poly unsaturated fatty acids and cholesterol, is a consequence of oxidative stress (van der Oost, et al., 2003). Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the capacity of the antioxidant defenses (Limón-Pacheco & Gonsebatt, 2009). ROS can be divided in radicals, for instance superoxide ion, hydroxyl and peroxyl groups, and non radicals such as hydrogen peroxide (Kohen & Nyska, 2002). Most of the radicals are very short lived and very reactive, since they are ready to donate or accept an electron, and the nonradical ROS have also a short half-life influenced by parameters such as pH and the presence of other species (Kohen & Nyska, 2002). Although less reactive ROS with longer half-life can be more toxic since they persist for long time allowing them to reach sensitive targets away from the local of production (Kohen & Nyska, 2002). The presence of ROS is harmful due to their ability to induce damage to nucleic acids, proteins, carbohydrates, and lipids (Limón-Pacheco & Gonsebatt, 2009). One of the most common ways to evaluate lipid peroxidation levels in animal tissues is to measure the thiobarbituric acid reactive substances (TBARS) (Yoshida, et al., 2012). The increase of LPO levels is also widely used in ecotoxicological studies.

Behaviour is defined as "an organism-level effect defined as the action, reaction or functioning of a system under a set of specific circumstances" (Hellou, 2011). Exposure to some environmental contaminants and their effects at sub-individual level can alter the behaviour of the organisms potentially leading to individual fitness decrease (Almeida, et al., 2010). Therefore, behaviour impairment has been also used as effect criteria in toxicity assays (Scott & Sloman, 2004). Behaviours that can be used as endpoints include avoidance/escape (e.g. Žižek & Zidar 2013), burrowing (e.g. Pan et al. 2012), feeding (e.g. Moreira et al. 2006), reproduction (e.g. Martins, Guimarães & Guilhermino 2013)

among others (Scott & Sloman, 2004; Hellou, 2011). These indicators had been pointed out as more sensitive than survival, growth or other more common endpoints after sub-lethal exposure (Atchison, et al., 1987; Hellou, 2011). The importance of studying behavioural endpoints is that they link biochemical and physiological processes, for instance, some behavioural alterations may be connected with endocrine disruption (Coltfelter, et al., 2004), and represent a whole organism response. Furthermore, changes in behaviour in response to chemical exposure can result in reduced fitness and survival of the individual in the natural environment, which can have a negative impact to the population as a whole (Kane, et al., 2005).

1.5. Objectives and structure of the thesis

The main goal of the present Thesis was to investigate the toxic effects of AuNP on juveniles of the estuarine fish *Pomatoschistus microps* (common goby), and the potential of microplastics presence and temperature increase (20ºC to 25ºC) to modulate the process. Four hypothesis were tested: (i) fish exposed to AuNP through the water uptake gold; (ii) AuNP (5 nm diameter) are toxic to *P. microps* juveniles at exposure concentrations in the ppb range; (ii) MP (polyethylene 1-5 µm spheres) interact with the effects of AuNP on *P. microps* juveniles; and (iii) the rise of temperature from 20ºC to 25ºC influences the effects of chemicals on *P. microps* juveniles.

P. microps was selected as model species for this study. This is a common species in estuaries and other coastal areas of Europe, with a wide range of distribution, from the Eastern Atlantic (e.g. Norway) to southern Portugal coasts and along the Mediterranean coasts (Gysels, et al., 2004). During the larval and early juvenile phases of its development, is mainly planktivorous, feeding mostly on copepods and other zooplankton species (Pockberger, et al., 2014). Therefore, its populations have an important function in controlling several zooplankton populations, avoiding the overdevelopment and decreasing their pressure over the phytoplankton (Pockberger, et al., 2014). During its entire life cycle, is a determinant prey to several higher predators, including fish species consumed by humans (Quintaneiro, et al., 2008). *P. microps* has been used as a model species in several types of studies. In Ecotoxicology, it is considered a very good bioindicator in relation to pollution (Monteiro, et al., 2007; Guimarães, et al., 2012) and a very convenient model organism for toxicity bioassays (Monteiro, et al., 2005; Oliveira, et al., 2013; Vieira, et al., 2008; Vieira, et al., 2009). Among other favorable characteristics, the species has a wide geographic distribution, in general is an abundant species, has a high reproduction rate, it can be found in sites with different degrees of pollution, is

relatively small, has a relatively short life cycle, and it is relatively easy to acclimate and maintain in the laboratory (Quintaneiro, et al., 2008).

AuNP were selected for this study because their use is likely to increase, particularly in biomedical applications, so, the levels of these particles in the environment is likely to increase as explained on section 1.2., thus, there is an urgent need for information about their effects on living organisms. Fluorescent polyethylene microspheres, hereafter indicated as MP, were selected for this study because polyethylene is one of the most produced polymers globally (PlasticsEurope, 2013) and one of the microplastics types more found in the marine environment and in wild organisms (Andrady, 2011; Galgani, et al., 2013). Additionally, these MP were found to be an adequate microplastic model in previous studies (Oliveira, et al., 2013).

The temperature of 20ºC and 25ºC were tested because they are ecologically relevant water temperatures in estuaries of South Europe (Almeida, et al., 2014; Gama, et al., 2014) in spring and summer. Furthermore, 20ºC is one of the most tested temperatures in bioassays with fish and therefore results obtained with other species are available for comparison, and 5ºC is a realistic rise of temperature in several estuaries in the next future according some global change scenarios for the next decades (IPCC, 2014).

The thesis is divided in four chapters: 1. Introduction; 2. Material and Methods; 3. Results and Discussion; 4. Conclusion. In the first chapter, the paradigms of the increasing environmental contamination (especially by nanomaterials and microplastics) and global warming are introduced, as well as how their effects on the biota (particularly fish) can be assessed. In the second chapter, the approaches and methods used during the experimental work are described, including the specific improvements done; in the third chapter the results obtained on the assays are presented and discussed and on the fourth chapter it is presented a general conclusion.

2. Materials and Methods

The experimental work was done in three main steps. A training period in fish toxicity testing with *P. microps* juveniles that are particularly sensitive to manipulation, in biomarkers determination, in spectrophotometry techniques used to determine the actual concentrations of toxicants in test media, in analyzing data from fish bioassay and in ecotoxicological parameters calculation. In the second phase of the study, a bioassay (hereafter indicated as preliminary AuNP bioassay) was carried out to have a first idea on the toxicity of AuNP to the model species and to select the concentration to be tested in the final bioassay. Finally, a comprehensive bioassay, hereafter indicated as multi-stressor bioassay, was done. Its goal was to assess the effects of AuNP on *P. microps* juveniles, their potential interaction with MP and the ability of temperature to modulate the toxicological process.

2.1. Tested substances and other chemicals

Spherical gold nanoparticles 5 ± 2 nm diameter, stabilized suspension in citrate buffer were purchased from Sigma-Aldrich (Germany). Fluorescent red polyethylene microspheres with a size range between 1 and 5 µm were purchased from Cospheric (USA). All the other chemicals were of analytical grade, obtained from Sigma–Aldrich (Germany), Bio-Rad (Germany) or Merck (Germany).

2.2. Fish Collection and Acclimation

The organisms used in this work were juveniles of the fish *P. microps*. They were collected from November 2013 to April 2014 on the Minho River estuary (41°52'43.74"N; 8°49'51.91"W) that has been considered a relatively low impacted estuary (Ferreira, et al., 2003). Fish from this estuary were used as test organisms in previous studies (Oliveira, et al., 2013; Vieira, et al., 2008; Vieira, et al., 2009). Juveniles were collected at low tide using a hand operated net. At the time and site of the capture, the water temperature, pH and dissolved oxygen levels (D.O.) were measured using an HACH® HQ40d (USA) probe. The fish were transported to the laboratory in the lowest time possible in thermally isolated boxes with water from the collecting site and with air supply (pump Nirox x-19, Malaysia).

The juveniles collected were gradually adapted to artificial sea water (ASW), prepared by dissolving sea salt (Tropic Marin® Sea Salt, Germany) in dechlorinated tap water for

human consumption, until reaching a salinity of 18 $g.L^{-1}$. All fish were maintained in 98 L glass aquaria filled with 68 L of ASW with continuous aeration and filtration (Eheim® classic, Germany), about 200 fish per aquarium, for at least two weeks before being used in bioassays. The water was partially renewed twice a week and the fish were fed daily with commercial fish food (Aquapex® tropicmix, Orni-ex, Portugal). Mortality was recorded daily and dead fish were removed as soon as noticed.

During this period, fish were maintained in a temperature and photoperiod controlled chambers (Bronson PGC 1400, The Netherlands) with a photoperiod of 16h light (L): 8h dark (D). The fish were acclimated to the appropriate temperature and other conditions according to their further use in the bioassays.

2.3. Bioassays

All the bioassays were performed generally according to the OECD guidelines for the testing chemicals nº 203 “Fish, Acute Toxicity Test” (OECD, 1992) with some modifications as indicated in the next sections. All the fish used as test organisms were acclimatized for at least two weeks to the conditions described in section 2.2 to the temperature and other conditions of the chambers (Bronson PGC 1400, The Netherlands) where the bioassays were carried out. In all cases, fish feeding was stopped 24h before each bioassay. Fish, randomly selected, were individually exposed for 96h in 1L glass beakers, previously washed with HNO_3 10% and then with distilled water, filled with 500 ml of test media. Air supply was continuously provided from an air compressor (Nitto Kohki ® médo, Japan) and distributed individually to the beakers. All the test beakers were sealed to prevent water evaporation. Fish were regularly observed along the day and dead fish were removed as soon as noticed. No food was provided to the fish during the exposure period. In all the assays, water temperature, pH, D.O. and salinity were measured at the beginning and at each 24h until the end of the bioassay. In all the bioassays, the effect criteria were: post-exposure predatory performance (hereafter indicated as predatory performance), and four sub-individual biomarkers, namely, the activity of the enzyme AChE as indicative of neurotoxicity; the activity of the enzymes EROD and GST as indicative of biotransformation alterations (phase I and II, respectively); GST was also used as indicative of oxidative stress; and increase of LPO levels as indicative of oxidative damage. Due to the high sensitivity of *P. microps* early juveniles to manipulation, the fish total length and weight were determined after the assessment of the predatory performance. Mortality was recognized through the lack of reaction after stimulation by a gentle touch with a plastic micropipette.

2.3.1. Preliminary AuNP bioassay

The fish used in this bioassay were captured in February 2014 and had a mean ± standard deviation of total length and weight of 2.08 ± 0.29 cm and 0.0809 ± 0.04 g respectively. Fish were acclimatized for at least two weeks to a temperature (20 ± 1ºC) and light cycle (16h light and 8h dark) controlled chamber (Bronson PGC 1400, Netherlands) where the bioassay was carried out. Two test concentrations of AuNP (1 $mg.L^{-1}$ and 0.5 $mg.L^{-1}$) were prepared by diluting the commercial AuNP suspension (69.40 $mg.L^{-1}$). The control medium contained only ASW prepared as previously indicated. Test medium was not renewed. In this assay were used 5 fish per treatment. Samples of 1 mL were taken from each beaker to determine the actual concentrations of AuNP in test media.

2.3.2. Multi-stressors bioassay

The fish used in this bioassay were collected in April 2014, and their mean and standard deviation (SD) of total length and weight were 2.24 ± 0.22 cm and 0.0955 ± 0.0234 g, respectively. The bioassay was carried out in two similar chambers (Bronson PGC 1400, Netherlands), one at 20ºC and the other at 25ºC. The fish tested at 20ºC were previously acclimatized at 20ºC, and those tested at 25ºC were previously acclimatized at 25ºC in the chambers used for the bioassay. Control medium was ASW (prepared as indicated in section 2.2). Test concentrations of AuNP were prepared by dilution of the commercial suspension (69.40 $mg.L^{-1}$) in ASW. A stock solution of MP (9.2 $mg.L^{-1}$) was prepared and the test solutions were obtained by dilution of the stock solution with ASW. At both temperatures, four treatments were used: a control (ASW); 0.2 $mg.L^{-1}$ of AuNP; 0.184$mg.L^{-1}$ of MP; 0.2 $mg.L^{-1}$ of AuNP + 0.184$mg.L^{-1}$ of MP. The exposure period was 96h and test media was renewed at 48h because the AuNP were found to precipitate in our experimental conditions in the preliminary bioassay with this substance. Fifteen fish, randomly selected, were used per treatment (9 for biomarkers determination and 6 for determination of the gold concentration in their body). Three replicates of each treatment without fish were included in the experimental design. Exposure conditions were the same as described in section 2.3. Samples for determination of MP and AuNP concentrations in test media and to characterize AuNP (sections 2.4 and 2.5) were collected. Additional test media samples for determination of their gold concentrations were collected at the same time, and the whole body of the 6 fish from each treatment reserved for gold determination

analysis. These samples were analysed by atomic absorption spectroscopy in the Department of Toxicology of the Faculty of Pharmacy of the University of Porto.

2.3.3. Post exposure predatory performance assay

After 96h of exposure, a post-exposure predator-prey assay was conducted to assess the performance of each fish in capturing/ingesting *Artemia* nauplii that are natural prey of *P. microps* juveniles. Before the assay (72h), *A. franciscana* cysts were put in ASW (37 $g.L^{-1}$) and left for 72h to hatch. Nauppii with about 24h old were used in the predator-prey assay. For this assay, each fish was placed in a predator-prey exposure chamber of 20 cm diameter containing 300 mL of ASW; after 5 minutes, 12 nauplii were added to the water. After 3 minutes, the fish was removed and the number of nauplii remaining in the chamber was counted. The number of prey captured/ingested by the fish was calculated as the difference between the initial number of prey introduced in the chamber (12) and the number remaining in the chamber after removing the fish. After this assay, all the fish were put back into their original exposure media and left to rest for 2 hours.

2.3.4. Preparation of biological material

After the 2h resting period, fish were sacrificed by decapitation under cold induced anesthesia. No other anesthetics were used to avoid influence on the biomarkers. From each fish, the head and the body were separately isolated on ice, and homogenized in ice cold phosphate buffer (0.1M; pH=7.2 and 7.4 for the head and body respectively) using an Ystral® D-79282 (Germany) homogenizer. The volume of buffer used was based on previous studies data on the expected content of protein in relation to fish size. Thus, for head samples the homogenization volume was 0.5mL for fish until 1.8 cm of total length and 1mL for fish longer than 1,8cm; for body samples the buffer volumes were between 0.7 and 3mL. Head homogenates were centrifuged for 3 minutes at 3300g (4ºC) using an Eppendorf® 5810R (Germany) centrifuge. The supernatant of each sample was carefully collected and stored at -80ºC for further determination of AChE activity. Each body homogenate was divided into three aliquots: one for LPO determination that was immediately stored at -80ºC; the others were centrifuged separately for 20 minutes at 10000g and 4ºC (Eppendorf® 5810R (Germany) centrifuge) to obtain the S9 fraction (post-mitochondrial); then each supernatant was carefully collected and stored at -80ºC until further use for GST and EROD activity determinations.

2.3.5. Biomarkers determination

The protein content of samples for AChE and GST activity determinations was normalized to 0.5 $mg.mL^{-1}$ as in Oliveira et al. (2013). Protein quantification was done by the Bradford method (Bradford, 1976) adapted to microplate (Frasco & Guilhermino, 2002). Briefly, protein concentration standards were prepared in the microplate (96-well microplate, Costar®, USA) by dilution of a standard solution of bovine γ-globulin (1 $mg.mL^{-1}$) with u.p. water, obtaining concentrations of 0 $mg.mL^{-1}$; 0.2 $mg.mL^{-1}$; 0.5 $mg.mL^{-1}$ and 1 $mg.mL^{-1}$ of protein (final concentrations in the microplate well) that are used for the calibration curve; 0.010 mL of each sample was pipetted to a well of the same microplate; 0.250mL of a Bio-Rad solution previously prepared by diluting 4 folds the commercial Bio-Rad solution was added to each microplate well; all the determinations were done in triplicate; after 15 minutes of mixing the optical density was read at 600nm in a BIO-TEK® powerwave 340 (USA) microplate reader. Because the protein standardization procedure was made by dilution after the first spectrophotometric reading aimed at standardize the protein content of the samples, the concentration of protein in each sample was determined again after the determination of its enzymatic activity to increase the accuracy when expressing the enzymatic activity in function of sample protein content.

AChE activity determination was performed in the supernatant of fish head homogenates previously prepared as described above (sub-section 2.3.4) using the Ellman's method (Ellman, et al., 1961) adapted to microplate (Guilhermino, et al., 1996). The cholinesterase activity measured in such samples in the conditions used per routine in our laboratory is mainly of AChE, as indicated by a previous study where the characterization of the enzymes present in *P. microps* fish head was made (Monteiro, et al., 2005). The Ellman´s technique is based on the formation of a yellow color whose optical density rises with the increase of acetylthiocholine degradation caused by the action of AChE present in the samples. Acetylthiocholine is hydrolyzed to acetate and thiocholine in the presence of the cromophore 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB); the thiocholine formed reacts with DTNB to produce the anion 5-thio-2-nitro-benzoic acid whose yellow color can be measured at 412 nm. Within a certain time interval, depending on the amount of enzyme present in the sample and other specific conditions of the assay, the AChE activity is proportional to the absorbance increase measured at 412nm and the enzymatic activity can be determined from the slope of the linear part of the absorbance increase curve. For the determination of AChE activity, a solution of acetylthiocholine (0.075 M) in u.p. water was prepared in advance in dark conditions and

maintained at 4ºC (also in dark conditions) for a maximal period of two weeks (solution A). A solution of DTNB (0.010M) with 0.018M of sodium bicarbonate in phosphate buffer (0.1M, pH=7.2) was also prepared in advance in dark conditions and kept at 4ºC (also in dark conditions) for a maximal period of two weeks (solution B). In the day of the assay and about half an hour before the enzymatic determinations, a reaction solution was prepared by adding 0.2 mL of the solution A and 1 mL of the solution B to 30 mL of phosphate buffer (0.1M, pH=7.2). For the analysis, 0.05 mL of head homogenate supernatant previously defrozen and gently mixed, were put into a well of a 96-well microplate (Costar® (USA), 0.320 mL of capacity) in triplicate. Then, 0.250 mL of the reaction solution was added. A well column was left empty to calibrate the microplate reader (BIO-TEK® powerwave 340) and a second well column was filled with the reaction solution only to be used as blank (degradation of acetylthiocholine without the enzyme action). The enzymatic activity was read at 412nm for 5 minutes. In the conditions used, the linear increase of the yellow color usually occurs for 2 minutes maximum within the 5 minutes interval (at the end of the time there is no linearity due to lack of substrate for the enzyme, and at the beginning the reaction may not be linear too because the mixing of substances in the reaction media may still be occurring). The enzymatic activity was calculated from the slope of the linear part of the reaction curve (absorbance vs. time), using the following formula:

$$A\left(U/mg_{protein}\right) = \frac{\frac{\Delta Abs}{\varepsilon . l} \times V_t}{m_{protein}(mg)} \times 10^9 \quad (1)$$

Where:

- A is the enzymatic activity in U per mg of protein in the sample, being U nmol of substrate consumed per minute;
- ΔAbs is the variation in absorbance per minute at 412 nm at their maximum slope;
- ε is the molar extinction coefficient of TNB ($13.6 \times 10^3 M^{-1} cm^{-1}$);
- l is the optical path;
- V_t is the total volume of the reaction mixture in litters;
- $m_{protein}$ is the amount of protein in the sample in milligrams.

LPO levels were analyzed using Ohkawa's procedure (Ohkawa, et al., 1979) which is based on the formation of thiobarbituric acid reactive substances (TBARS) measured at 535nm. For that, a solution of trichloroacetic acid (TCA) at 12% was prepared in u.p.

water (solution A); another solution of thiobarbituric acid (TBA) at 0.73% was prepared in pre-heated u.p. water in order to dissolve the TBA (solution B); and a solution of Tris-HCl (0.060 M) with diethylenetriaminepentaacetic acid (DTPA) ($0.1x10^{-3}$ M) was prepared in u.p. water and its pH stabilized at 7.4 with sodium hydroxide (NaOH) solution C. Then, tubes containing 0.2 mL of body homogenate (0.2mL of phosphate buffer 0.1M, pH=7.4 for blanks) were incubated in a water bath at 100ºC for 60 minutes in the presence of 1mL of solution A, 0.800 mL of solution C and 1mL of solution B. After the incubation, samples were centrifuged at 14000g for 5 minutes at 25ºC, their optical density was measured at 535nm in a Jasco V-630 spectrophotometer and their protein content determined as previously indicated. LPO levels were calculated and expressed as nmol of TBARS formed per mg of protein according to the following formula:

$$LPO\left(U/mg_{protein}\right) = \frac{\frac{Abs_{535nm}}{\varepsilon.l} \times V_t}{m_{protein}(mg)} \times 10^9 \quad (2)$$

Where:

- LPO refers to the lipid peroxidation levels in U per mg of protein in the sample, being U nmol of TBARS formed per minute;
- Abs_{535nm} is the absorbance value read at 535nm
- ε is the molar extinction coefficient of TBARS (ε=1.56 x 10^5 $M^{-1}cm^{-1}$).
- l is the optical path;
- V_t is the total volume of the reaction mixture in litters;
- $m_{protein}$ is the amount of protein in the sample in milligrams.

GST determination was done according to the method of Habig *et al.* (1974) (Habig, et al., 1974) adapted to microplate (Frasco & Guilhermino, 2002). Briefly, a solution of 1-chloro-2.4-dinitrobenzene (CDNB) 0.060 M was prepared in ethanol (100%) and kept in ice (solution A); a solution of glutathione (GSH) 0.010 M was prepared in phosphate buffer (0.1M, pH=6.5) and kept in ice (solution B). A reaction solution was prepared immediately before usage by adding 1.5 mL of the solution A and 9 mL of the solution B to 49.5 ml of phosphate buffer (0.1M, pH=6.5). Then, 0.250 mL of the reaction solution was added to 0.050mL of the body samples (S9 fraction supernatants) in a 96-well microplate; 8 wells containing only 0.050 mL of phosphate buffer were used as blank. The kinetic of the reaction was read at 340nm for 5 minutes at 340nm on a BIO-TEK® powerwave 340 microplate reader, and the enzymatic activity was determined from the

slope of the linear part of the absorbance *versus* time curve. This method is based on the conjugation of CDNB with the thiol group of GSH, a reaction that is catalyzed by the enzymes GST present in the samples under analysis. This conjugation reaction leads to an increase of the absorbance at 340nm. After protein determination, GST activity was calculated using equation 1 using the molar extinction coefficient of the conjugate (ε= $9.6\times10^{3}M^{-1}cm^{-1}$) and expressed as nmol of conjugate formed per minute per mg of protein.

EROD activity was evaluated in body supernatant pooled samples (3 fish body homogenates each) by measuring the rate of deethylation of ethoxyresorufin into resorufin catalyzed by EROD in the presence of nicotinamide adenine dinucleotide phosphate (NADPH). The amount of resorufin formed was measured fluorometrically. Briefly, a reaction solution was prepared containing 1.5 mL of a stock solution of ethoxyresorufin (410.85 mM) prepared in u.p water and 49.5 mL of phosphate buffer (0.1 M, pH=7.4); 1mL of the reaction solution was added to 0.100 mL of the pool of fish body S9 supernatants previously obtained (sub-section 2.3.4) and then 0.010 mL of a NADPH solution in phosphate buffer 0.1 M, pH= 7.4 (11.15 mM) was added to the mixture; blanks contained 0.100 mL of phosphate buffer (0.1 M, pH=7.4) instead of fish body homogenate. The kinetic of the reaction was measured on a Jasco FP-6200 spectrofluorometer for 6 minutes, the wavelengths of excitation and emission were 530nm and 585nm, respectively. Protein levels were quantified after the enzymatic assay and the results were expressed as pico moles (pmol) of resorufin formed per minute per mg of protein using equation 3:

$$A\left(U/mg_{protein}\right) = \frac{|resorufin\ formed| \times V_t}{m_{protein}(mg)} \times 10^3 \quad (3)$$

Where:

- A is the enzymatic activity in U per mg of protein in the sample, being U pmol of substrate consumed per minute;
- $|resorufin\ formed|$ is the concentration of resorufin formed per minute,;
- V_t is the total volume of the reaction mixture in litters;
- $m_{protein}$ is the amount of protein in the sample in milligrams.

2.4. AuNP characterization

The characterization of AuNP was done before and along the bioassay in order to estimate the concentration, size and aggregation state and to try to evaluate how these properties change under the assays' conditions. Direct estimate of size, shape and aggregation state from UV-Vis spectra was performed based on some methods suggested on literature (Haiss, et al., 2007; Amendola & Meneghetti, 2009). Equation (4) deduced by Haiss *et al.* (2007) to estimate the size of the nanoparticles through the UV-Vis spectra which is based on the linear relationship between the ratio of the absorbance at the peak and at 450nm and the diameter of the nanoparticles.

$$d = \exp\left(B_1 \frac{A_{spr}}{A_{450}} - B_2\right) \quad (4)$$

Where:

- B1 and B2 parameters were experimentally deduced by the authors and are 3.00 and 2.20 respectively.
- A_{spr} is the absorbance value at the peak (surface plasmon resonance) and A_{450} is the absorbance value at a wavelength of 450nm.

The method presented by Amendola & Meneghetti (2009) based on the Mie model for spheres and Gans model for spheroids was also adopted to estimate the shape (% of spheres) of the nanoparticles.

Before the bioassays, three independent AuNP suspensions with a concentration of 20mg.L^{-1} were prepared by dilution of the commercial suspension in ASW; similar suspensions were prepared in u.p. water. Each suspension was then serially diluted (1:2 v/v) to obtain seven additional suspensions (10.00mg.L^{-1}; 5.00mg.L^{-1}; 2.50mg.L^{-1}; 1.25mg.L^{-1}; 0.625mg.L^{-1}; 0.3125mg.L^{-1}; 0.156mg.L^{-1}). Full UV-Vis spectra of these suspensions were measured and used to estimate the size of the AuNP in ASW suspensions using the method of Haiss *et al.* 2007 hence, testing the adequacy of using this spectrophotometer procedure to estimate size of AuNP in ASW. The absorbance at 530 nm (the peak of absorbance in our experimental conditions) at 0h of the previously prepared suspensions was recorded. The correlation between the optical density values at 530 nm (the peak of absorbance in our experimental conditions) and AuNP nominal concentrations was investigated using the Pearson's correlation coefficient. A significant correlation was found and a linear regression model was adjusted to the data using the

absorbance as independent variable and AuNP nominal concentrations as dependent variable. The equation of the model was used to determine the actual concentrations of AuNP in test media from the corresponding absorbance values at 530 nm.

Suspensions with a nominal concentration of 20mg.L^{-1}; 5mg.L^{-1} and 1.5mg.L^{-1} were prepared in ASW and let for 48h at 20ºC and 25ºC in the same conditions of the multi-stressors bioassay. Their full UV-Vis spectra were determined immediately after preparation (0h) and at 24h and 48h using a Jasco V-630 spectrophotometer. The objective of this was to investigate if the properties of AuNP change over time in our test media in the conditions of the multi-stressor bioassay.

The decay of AuNP along the bioassays was investigated by measuring the absorbance of test media at 530 nm at the beginning of the assay, at the time of medium renewal (48h) and at each 24h in both fresh and old media. Three replicates of each treatment without fish were included in the experimental design (at both 20 and 25ºC) and treated equally as the treatments containing fish to investigate potential biological influences on AuNP decay. The decay of AuNP (in the original form) during 48h (time renewal interval) was calculated as: %Decay = 100-(Abs_{old} / Abs_{fresh})*100.

2.5. Determination of microplastic concentrations and decay

The determination of the MP concentrations in test media of the multi-stressors bioassay was performed by spectrofluorimetry. Three independent suspensions of MP with a concentration of 12 mg.L^{-1} were prepared in ASW. Each solution was serial diluted (1:2 v/v) to obtain 10 additional suspensions in a final range of concentrations between 12 mg.L^{-1} and 0.012mg.L^{-1}. The suspensions with concentrations between 1.5mg.L^{-1} and 0.012mg.L^{-1} were selected for the calibration curve aimed at further determining the MP concentrations in test media during the bioassay. The selection of the concentrations to be used was done in order to have the nominal concentration of microplastics to be used on the assays (0.184mg.L^{-1}) in the middle of the curve to allow a more accurate calculation of the actual concentrations on the test media. The fluorescence was read (excitation wavelength of 470nm and emission wavelength of 588nm). The correlation between the fluorescence readings and MP nominal concentrations was analyzed by the Pearson correlation coefficient (r) after checking the normality of the data. The decay of MP in test media was investigated by comparing the fluorescence readings in the fresh media (0h) with the fluorescence of 24h and 48h old media: %Decay = 100 - (F_{old}/ F_{fresh})*100. Three replicates of each treatment without fish were included in the

experimental design (at both 20 and 25ºC) and treated equally as the treatments containing fish to investigate potential biological influences on MP decay.

2.6. Determination of the bioconcentration of gold on fish body

The bioconcentration factor (BCF) were calculated to assess if the *P. microps* bioconcentrate gold in their body, after 96h of exposure to AuNP in the presence and absence of MP. It were used the gold concentrations in 48h old media and in the fish body determined by chemical analysis. The BCF was also calculated through the concentration of AuNP calculated by spectrophotometry. The formula used to calculate the BCF was: BCF = concentration of gold in the fish body / concentration of gold in ASW.

2.7. Statistical Analysis

The results obtained in each assay with AuNP for the different treatments were tested for normality using Shapiro-Wilk test and for homogeneity of variances using the Levene homogeneity test. Predatory performance percentages were arcsine transformed (Zar, 1996). For the other parameters, appropriate data transformations were performed when the requirements of normal distribution and/or homogeneity of variances were not achieved.

For each parameter of the multi-stressors bioassay, a two-way ANOVA with interaction was carried out to investigate differences among treatments, temperatures and the potential interaction between them. A post-hoc Tukey test was performed when significant differences between treatments were detected by the two-way ANOVA. One-way ANOVA were performed to investigate if there were significant differences between treatments on the assays made at each temperature individually. Again, a post-hoc Tukey test was performed when significant differences between treatments were found by the one-way ANOVA. If the two-way ANOVA performed detected differences between temperatures, the effect of temperature variation on each treatment was assessed performing a Student's *t*-test, since the analysis performed on the two-way ANOVA groups every replicate at each temperature and compares the means and here we wanted to compare the means of each treatment obtained at the two temperatures tested. The SPSS 22.0 statistical package was used for all the analysis and the significance level was 0.05.

3. Results and Discussion

3.1. UV-Vis characterization of AuNP

The UV-Vis spectra of AuNP suspensions in u.p. water and ASW are shown in Figure 1. In u.p. water, the highest absorbance was recorded at 520 nm which is in good agreement with the supplier information and with previous published data indicating that suspensions of non aggregated gold nanoparticles within this size range display an absorbance pick at this wavelength (Amendola & Meneghetti, 2009). In ASW (salinity 18 $g.L^{-1}$), the corresponding absorption peak appears at 530 nm indicating that the ASW can change the optical properties of the AuNP probably due to the difference on the ionic strength between ASW and u.p. water.

The diameter of the AuNP present in u.p. water and ASW suspensions using the formula of Haiss *et al.* (Haiss, et al., 2007) (equation 4, section 2.4) are indicated in Table 2. The mean and standard deviation (SD) of all the suspensions in u.p. water and in ASW were 4.23 ± 0.49 and 4.38 ± 1.15 nm respectively (Table 2). The diameter of the AUNP indicated by the manufacturer is 5 ± 2 nm (mean ± SD). Therefore, the diameter calculated from the equation 4 is within this range. Thus, the method can be used to estimate the diameter of AuNP in ASW in the range of concentrations 0.312 – 20 $mg.L^{-1}$.

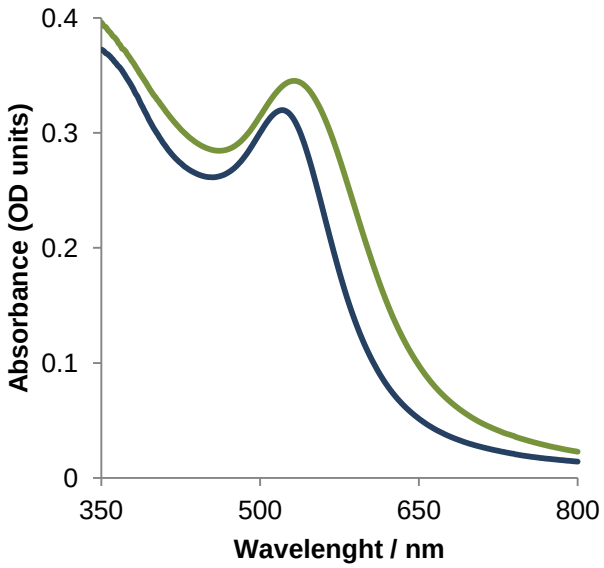

Figure 1. Representative UV-Vis spectra of 5 ± 2 nm (mean ± standard deviation) gold nanoparticles in u.p water (blue line) and artificial salt water (18 $mg.L^{-1}$ of marine salt) (green line) OD – Optical density

Table 2. Diameter of the gold nanoparticles (AuNP) in ultra-pure (u.p.) water and artificial saltwater (ASW) suspensions calculated (three replicate suspensions for each concentration) using the equation 4 of section 2.4 (Haiss, et al., 2007). SD – standard deviation.

AuNP concentration ($mg.L^{-1}$)	Calculated diameter / nm			
	u.p. water		ASW	
	Individual replicate suspension	Mean ± SD	Individual replicate suspension	Mean ± SD
0.3125	4.96 3.85 5.28	4.70 ± 0.75	5.87 4.66 3.99	4.84 ± 0.95
0.625	2.59 3.83 4.12	3.52 ± 0.81	4.02 4.67 8.97	5.89 ± 2.70
1.25	4.14 4.10 4.06	4.10 ± 0.04	3.72 3.77 3.76	3.75 ± 0.03
2.5	4.32 4.23 4.41	4.32 ± 0.09	4.02 3.89 3.80	3.90 ± 0.11
5	4.27 4.30 4.33	4.30 ± 0.03	4.04 3.92 3.88	3.95 ± 0.09
10	4.31 4.34 4.34	4.33 ± 0.02	4.15 4.07 4.04	4.09 ± 0.06
20	4.31 4.35 4.33	4.33 ± 0.02	4.28 4.22 4.17	4.22 ± 0.05
Overall mean ± SD	4.23 ± 0.49		4.38 ± 1.15	

The calibration curve data are shown in Figure 2. A positive and significant correlation ($N = 15$, $r = 0.998$, $p < 0.05$) between the absorbance and the corresponding AuNP nominal concentration was found. The linear model fitted to the data, using absorbance as independent variable (because during the bioassays, we will calculate the actual concentration of AuNP from the absorbance values) and nominal concentration as dependent variable, is: AuNP concentration ($mg.L^{-1}$) = 74.226 x absorbance (OD units) +

0.041. This model was used to determine the concentration of 5 nm AuNP along the bioassays.

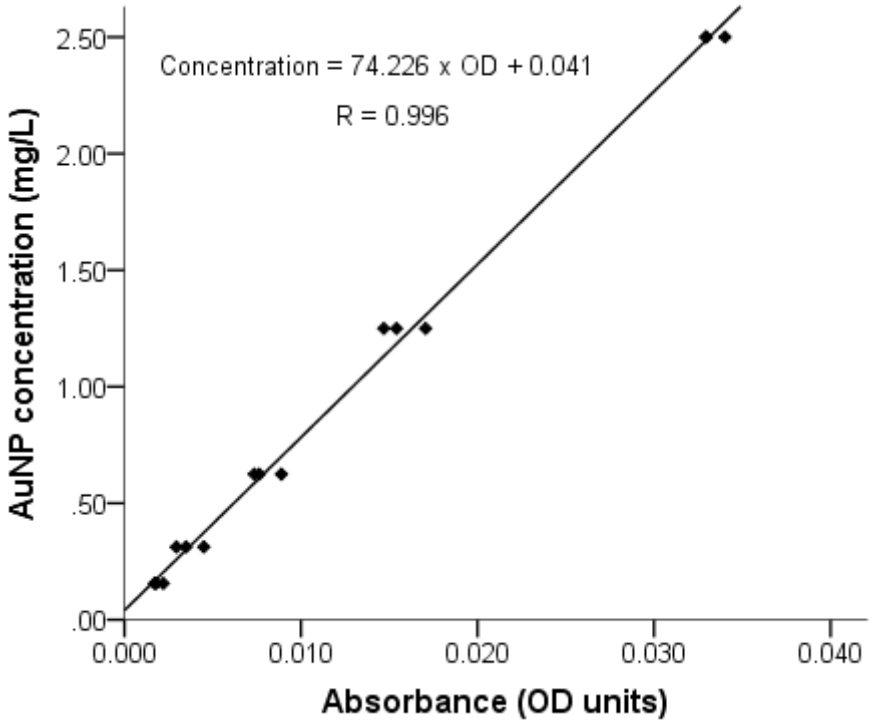

Figure 2. Calibration curve of the 5nm gold nanoparticles (AuNP) in ASW (18g.L^{-1} of salinity) with the linear model fitted to the data, using absorbance as independent variable and the nominal concentration of the AuNP suspensions as dependent variable, according its further use to determine the actual AuNP in test media of the bioassays. R – Coefficient of determination. OD – optical density.

3.2. Calibration curve to determine the MP actual concentrations in test media

The MP fluorescence *versus* concentration curve is shown in Figure 3. A positive and significant correlation (N = 24, r = 0.992, $p < 0.05$) was found. The linear model fitted to the data was: MP concentration (mg.L^{-1}) = 0.014 x Fluorescence - 0.108. Therefore, in the range of concentrations between 0.012 mg.L^{-1} and 1.50 mg.L^{-1}, this model can be used to determine the actual concentrations of MP in test media during the bioassays.

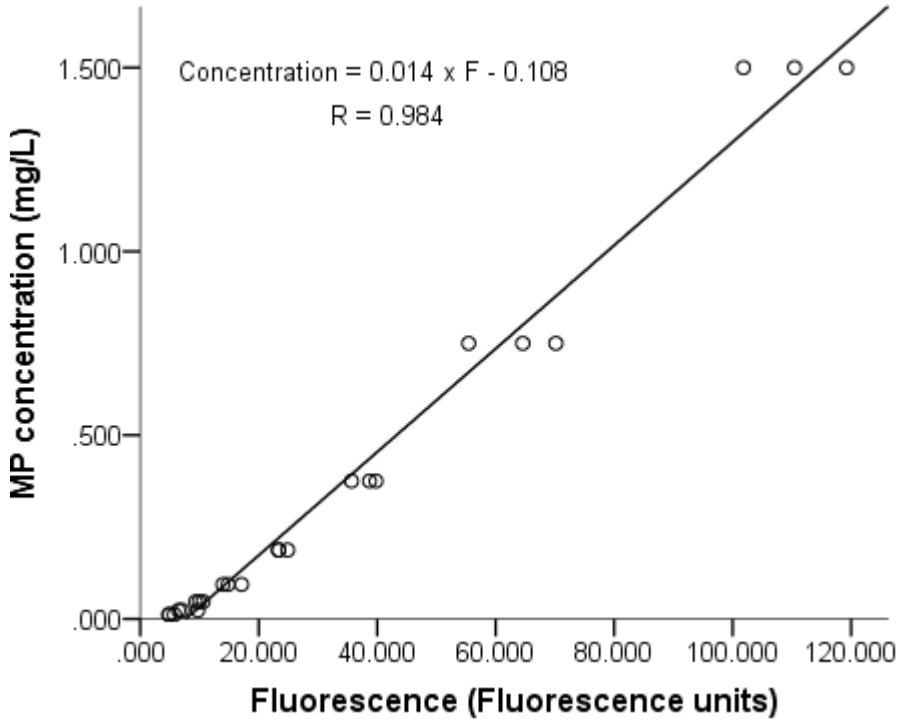

Figure 3. Calibration curve for the fluorescent red polyethylene microspheres (MP) in ASW (18g.L^{-1} of salinity) with the linear model fitted to the data, using the fluorescence values as independent variable and the nominal concentration of the MP suspensions as dependent variable, according its further use to determine the actual MP in test media of the bioassays. R – Coefficient of determination.

3.3. Preliminary assay with AuNP

The actual concentrations of AuNP determined from the absorbance readings of test media along the bioassay using the spectrophotometric method previously validated for ASW and the model fitted to data (section 3.1) are indicated in Table 3. At the beginning of the bioassay, the AuNP actual concentrations were 0.930 ± 0.016 mg.L^{-1} and 0.489 ± 0.011 mg.L^{-1} corresponding to nominal concentrations of 1.0 and 0.5 mg.L^{-1} respectively. Therefore, no significant deviations of actual concentrations relatively to nominal ones were found. The decay of AuNP (5 nm) along the bioassay ranged from 8% and 45%, with higher decays for the nominal concentration of 1.0 mg.L^{-1} than for the nominal concentration of 0.5 mg.L^{-1}, and was time-dependent. At 24h, the decay was 8 and 9 %, whereas at 48h was 14 and 30%, for the lowest and highest concentrations of AuNP tested respectively. Considering the results presented on Table 3 and to prevent a considerable decrease of the exposure concentrations along the assay, it was decided to renew the test media at 48h in further bioassays with AuNP.

The mortality recorded in fish after 96 h of exposure to different treatments is shown in Table 4. The mortality in the control group exceeded 10%. These results indicate that fish

were not in an adequate health status, and thus the bioassay cannot be considered valid (OECD, 1992). Fish exposed to 0.5 and 1.0 mg.L^{-1} of AuNP had a mortality of 60 and 80%, respectively. Although these findings deserved some reserve because a high mortality was recorded in the control group, they suggest that 0.5 and 1.0 mg.L^{-1} of AuNP may be lethal concentrations to *P. microps* juveniles. Because the objectives of the present study included the investigation of the effects of the tested substances on the predatory behaviour that needs to be determined in fish alive, it was decided to test a lower AuNP concentration (0.2 mg.L^{-1}) in further bioassays.

Table 3. Actual concentrations of AuNP on the test media and its percentage of decay (% Decay). The values are presented as means ± standard errors of the mean.

Nominal AuNP concentration / mg.L^{-1}	Time	N	Actual concentration/ mg.L^{-1}	%Decay
1.00	0h	5	0.930±0.016	0
	24h	3	0.844±0.041	9
	48h	3	0.652±0.027	30
	72h	3	0.642±0.103	31
	96h	3	0.512±0.042	45
0.50	0h	5	0.489±0.011	0
	24h	5	0.452±0.026	8
	48h	5	0.423±0.050	14
	72h	4	0.387±0.053	21
	96h	4	0.355±0.072	27

Table 4. Registered mortality (%) on the preliminary assay with AuNP after 96h.

	Control	AuNP concentration / mg.L^{-1}	
		0.50	1.00
% Mortality	40%	60%	80%

3.4. Multi-stressors bioassay

3.4.1. Behaviour of AuNP in ASW, actual concentrations and decay

The UV-vis spectra of AuNP in ASW (nominal concentration of 20 mg.L^{-1}), in the absence of fish, maintained at 20º and 25ºC in the conditions of the bioassay are shown in Figure 4. At both temperatures, the aborbance peak shifted to longer wavelenghts over time: at 20ºC, from 530 nm at 0h to 540 nm at 24h; and 550nm at 48h; at 25ºC, from 537

nm at 0h, to 552 nm at 24 h, and 582nm at 48h. This suggests alteration of the properties of AuNP over 48h.

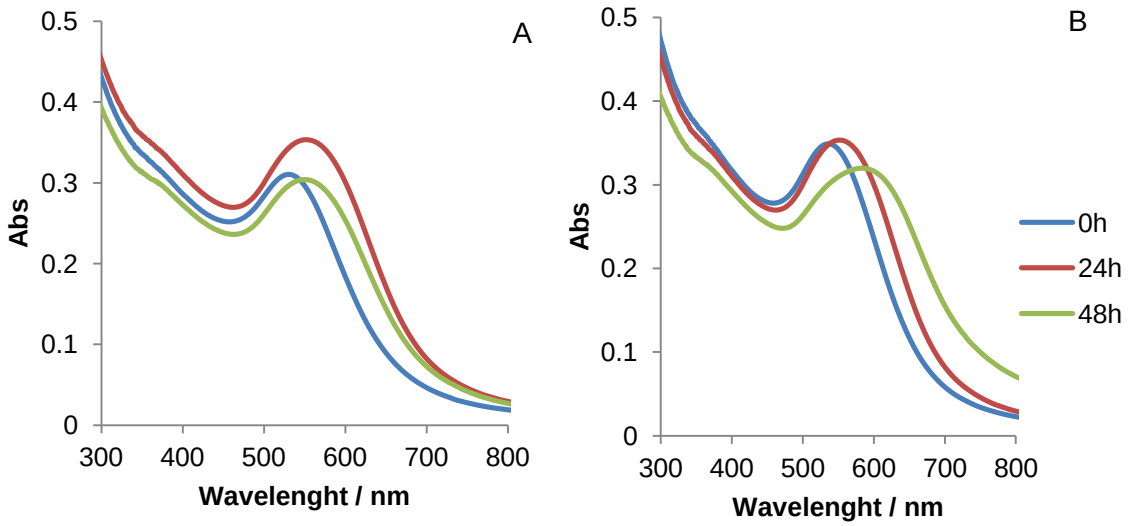

Figure 4. UV-Vis spectra of 5nm of diameter AuNP in ASW (nominal AuNP concentration of 20mg.L^{-1}) at 0, 24 and 48 hours at 20ºC (A) and 25ºC (B) (in the absence of fish).

The estimatiom of the size of the particles present using the equation (4) (Haiss, et al., 2007) showed in Table 5, indicates an increase of the diamenter of the AuNP of 15 % at 20ºC and of 3% at 25ºC. Moreover, at 20ºC, the shape of the particles, determined using the method proposed by Amedola & Meneghetti (2009) changed from 100% of spheres to 85% at 24h, remaining constant until the end of the medium renewal time (48h). Therefore, both size and shape changes occurred and these alterations may explain (or at least contribute to) to the shift of the absorbance peack recorded. The increase of AuNP size and shape alterations observed may be due to aggregation, a phenomena that can result in a higher percentage of non spherical nanoparticles in suspension (Amendola & Meneghetti 2009). Larger particles are also formed by aggregation and the absorbance peak of AuNP suspensions shifts to longer wavelengths as particle size increase (Pedersen & Duncan, 2005). The results presented in Figure 4 and Table 5, also show some differences on AuNP behaviour maintained at distinct temperatures. First, the shift of the peak at 25ºC was more pronounced than at 20ºC. Secondly, at time 0h, all the particles were spheres in the suspenctions maintained at 20ºC, while only 85% had this shape at 25ºC. Finally, the size of the AuNP was higher at 25ºC than at 20ºC in the time periods. These findings indicate that temperature influences the behaviour of AuNP in saltwater, by increasing their aggregation, leading to the formation of larger and more

irregular particles. These findings are in good agreement with the results from the literature on AuNP (Lapresta-Fernández, et al., 2012) as well as for other type of nanoparticles (Chen, et al., 2012).

Table 5. Size and percentage of spherical AuNP suspended in ASW (nominal concentrations of 20; 5 and 1.5 mg.L^{-1}) without fish maintained at 20ºC and 25ºC under conditions similar to those used in the multi-stressor bioassay. The size was estimated using the equation 4 (Haiss, et al., 2007) and the shape (% of spheres) was determined according to Amendola & Meneghetti (2009) method.

Temperature	Time	N	Size / nm	% Spheres
20ºC	0h	3	4.43	100
	24h	3	4.83	85
	48h	3	5.09	85
25ºC	0h	3	4.71	85
	24h	3	5.47	85
	48h	3	4.84	85

The actual concentrations of AuNP determined in fresh test media (immediately after its preparation and before the introduction of fish in the beakers) of the multi-stressors bioassay are indicated in Table 6.

Table 6. Nominal and actual concentrations of 5 nm gold nanoparticles (AuNP) in fresh test media at 20ºC and 25ºC. The actual concentrations were determined by spectrophotometry according to the model of Figure 2 and are expressed as the mean of 6 replicates (N) with the corresponding standard error within brackets. The deviation was calculated as: Deviation (relatively to nominal AuNP concentration) = (actual AuNP concentration * 100 / nominal AuNP concentration) - 100. Conc. – concentration; Treat. – Treatment; N - number of samples analyzed.

Temperature (ºC)	Nominal AuNP conc. (mg.L^{-1})	Treatment	N	Actual AuNP conc. (mg.L^{-1})	Deviation (%)
20ºC	0.200	AuNP only	6	0.337 (± 0.061)	69
	0.200	AuNP + MP	6	0.321 (± 0.013)	61
25ºC	0.200	AuNP only	6	0.258 (± 0.019)	30
	0.200	AuNP + MP	6	0.278 (± 0.025)	39
Overall mean	0.200		24	0.299 (± 0.018)	50

Contrary to the findings obtained in the preliminary bioassay, considerable deviations from the nominal concentration (0.2 $mg.L^{-1}$), ranging from 30 to 69%, were found. They may be due to several reasons, including the difficulty in preparing nanoparticles homogenous suspensions with low AuNP concentrations; aggregation or reaction with ASW (because the concentration is relatively low, these processes may be relatively more relevant than at higher concentrations); because the concentration interval used for the calibration curve was from 0.156 $mg.L^{-1}$ to 2.5 $mg.L^{-1}$, the model may not fit so well at the lower range of the distribution; and lack of sensitivity of the spectrophotometric method at low concentrations (e.g. 0.2 $mg.L^{-1}$). These deviations highlight the difficulties in testing AuNP and other nanoparticles, particularly at low concentrations and using saltwater as test media.

The decay of 5 nm AuNP along the bioassay determined directly from the absorbance values to avoid potential bias due to a decreased fit of the model at low concentrations is shown in Table 7. In the absence of fish, no decay of AuNP in test media was found after 24h. However, after 48h, a decay ranging from 36 % to 46% was found. The 5ºC rise of temperature increased the percentage of AuNP decay, and the presence of MP slightly decreased the decay of AuNP. The ionic strength of the ASW may be the main responsible for this observation, since the electrostatic repulsion between particles is reduced with increasing concentration of electrolytes (Macpherson, et al., 2012) allowing more contact between particles which leads to particle aggregation like it happens with other metallic nanoparticles in high ionic strength media (French, et al., 2009; Chekli, et al., 2013).

The AuNP decay in test beakers with fish (Table 8) was higher than in test beakers without fish (Table 7). These findings indicate that AuNP were taken up by fish. As found in test beakers without fish, the AuNP decay increased with temperature but the difference between temperatures was higher: 27% of difference in the presence of fish, 8% without fish in test media without MP. Thus, temperature may have increased the uptake of AuNP by fish. Other explanation can be that the fish metabolism and excretion rate at 25ºC may be higher (Clarke & Johnston, 1999; Nerici, et al., 2012) and its excretion products may interact with the AuNP and provoke their aggregation and/or precipitation or change their optical properties At 20ºC, the decay of AuNP was higher in the presence of MP (Table 8), whereas in test media without fish (Table 7) the decay was about the same; at 25ºC, in the presence of fish, the decay was almost the same in test media with and without MP. These results suggest that fish influence the interactions between AuNP and MP and that the process is modulated by temperature.

Table 7. Decay of 5 nm gold nanoparticles (AuNP) in artificial sea water (ASW) used as test media in the absence of fish during 48h (time of medium renewal of the bioassays) carried out at 20ºC and 25ºC. The values are the means with the corresponding standard errors within brackets. T – Temperature; Treat. – treatment; N - number of samples analyzed. The decay was calculated as: decay = 100-(Abs old media/ Abs 0h)*100

T	Treat.	N	Abs 0h	Abs 24h	Abs 48h	Decay (%) 0h-24h	Decay (%) 0h-48h
20ºC	AuNP only	3	0.0024 (± 0.0002)	0.0024 (± 0.0002)	0.0015 (± 0.0003)	0	38
	AuNP + MP	3	0.0025 (± 0.0006)	0.0025 (± 0.0003)	0.0016 (± 0.0002)	0	36
25ºC	AuNP only	3	0.0026 (± 0.0001)	0.0029 (± 0.0002)	0.0014 (± 0.0000)	0	46
	AuNP + MP	3	0.0030 (± 0.0001)	0.0032 (± 0.0008)	0.0018 (± 0.0004)	0	40

Table 8. Decay of AuNP over time (after 24 and 48h) in the test beakers containing fish in the presence and absence of microplastics at 20ºC and 25ºC. The decay was determined from the absorbance (optical density units - OD) relatively to the absorbance of freshly prepared AuNP test media (0h) through the formula: decay = 100-(Abs old media/ Abs 0h)*100. The values are the means of determinations in different test beakers (N) with the standard error within brackets. T – Temperature; Treat. – Treatment; N - number of samples analyzed.

T	Treat.	N	Abs 0h	N	Abs 24h	N	Abs 48h	Decay (%) 0h-24h	Decay (%) 0h-48h
20ºC	AuNP only	6	0.0040 (± 0.0008)	30	0.0032 (± 0.0004)	30	0.0028 (± 0.0003)	21	30
	AuNP + MP	6	0.0038 (± 0.0002)	30	0.0029 (± 0.0003)	30	0.0019 (± 0.0002)	25	49
25ºC	AuNP only	6	0.0029 (± 0.0003)	29	0.0020 (± 0.0003)	29	0.0013 (± 0.0002)	33	57
	AuNP + MP	6	0.0032 (± 0.0003)	29	0.0018 (± 0.0002)	29	0.0014 (± 0.0002)	43	56

3.4.2. MP actual concentrations and decay

The actual concentrations of MP calculated through the model of Figure 3 are shown in Table 9. The deviations of actual concentrations from the nominal ones ranged from 2 to 23%. As the AuNP, MP are in suspension in the test media and thus some of the

factors that may have contributed to these findings previously discussed for AuNP may also apply here. Because only one treatment slightly exceeded the maximum decay limit of 20% recommended by OECD guidelines for acute testing with juvenile fish (OECD, 1992), the concentration of MP will be hereafter indicated as 0.184 mg.L^{-1}.

Table 9. Nominal and actual concentrations of microplastics in fresh media at 20ºC and 25ºC. The actual concentrations were determined by spectrofluorimetry according to the model of Figure 3 and expressed as the mean of the replicates ± S.E.M. The deviation was calculated as: Deviation (relatively to nominal MP concentration) = (actual MP concentration * 100 / nominal MP concentration) – 100. Nom. – nominal concentration; Conc. – concentration; Treat. – Treatment; N - number of samples analyzed. S.E.M. – standard error of the mean.

Temperature	Nominal Conc. (mg.L^{-1})	Treat.	N	Actual Conc. (mg.L^{-1})	Deviation (%)
20ºC	0.184	MP only	6	0.226 (± 0.004)	23
	0.184	MP + AuNP	6	0.188 (± 0.016)	2
25ºC	0.184	MP only	6	0.211 (± 0.003)	15
	0.184	MP + AuNP	6	0.150 (± 0.008)	18
Overall Mean	0.184		24	0.194 (± 0.007)	5

Table 10. Decay of MP in artificial sea water (ASW) used as test media in the absence of fish during 48h (time of medium renewal of the bioassays) carried out at 20ºC and 25ºC. The values are presented as means ± standard errors within brackets. T – Temperature; Treat. – Treatment; N - number of samples analyzed; F- Fluorescence (F units).

T	Treat.	N	F 0h	F 24h	F 48h	Decay (%) 0h-24h	Decay (%) 0h-48h
20ºC	MP only	3	22.221 (± 1.08)	19.624 (± 3.548)	15.953 (± 1.095)	12	28
	MP + AuNP	3	22.235 (± 1.285)	14.768 (± 0.567)	16.678 (± 1.822)	34	25
25ºC	MP only	3	20.909 (± 0.400)	15.860 (± 0.491)	14.470 (± 0.341)	24	31
	MP + AuNP	3	24.443 (± 0.459)	16.173 (± 0.110)	14.340 (± 0.264)	34	41

In the absence of fish the decay of MP after 48h in ASW ranged between 25% and 41% (Table 10). This observation may be due to the fact that with increasing salt concentration MP tend to be separated from the water column (Cole, et al., 2011). Additionally, the ASW may change the fluorescence properties of the MP reducing the fluorescence value read by the spectrofluorometer. On the treatment with MP only the differences on the decay value at different temperatures was only 3% whereas in the treatment with MP + AuNP at 25ºC the registered decay was 16% higher than the decay at 20ºC.

The decay of MP during the assays (Table 11) was also significant although slightly lower than the decay registered for the AuNP. In the presence of AuNP the registered decay of MP was higher than in the treatments with MP only which may be due to interactions between the two types of particles which can induce the precipitation of microplastics and/or change the fluorescence absorption/emission of the microplastic spheres thus reducing the fluorescence value read by the spectrofluorometer. Similarly as for AuNP, the decay of microplastics after 48h at 25ºC was higher than at 20ºC, then again, the higher fish uptake and excretion of metabolites which can be adsorbed to the hydrophobic surface of microplastics and may change their fluorescence properties and/or inducing precipitation are the more plausible justifications.

Table 11. Decay of microplastics over time (after 24 and 48h) in the test beakers containing fish in the presence and absence of AuNP at 20ºC and 25ºC. The decay was determined from the fluorescence (F) relatively to the absorbance of freshly prepared AuNP test media (0h) through the formula: decay = 100-(F old media/ F 0h)*100. The values are presented as means ± standard errors of the mean. T – Temperature; Treat. – Treatment; N - number of samples analyzed.

T	Treat.	N	F 0h	N	F 24h	N	F 48h	Decay (%) 0h-24h	Decay (%) 0h-48h
20ºC	MP only	6	23.867 (± 0.254)	18	19.973 (± 0.333)	18	18.359 (± 0.604)	16	23
	MP + AuNP	6	21.139 (± 1.17)	30	14.823 (± 0.272)	30	13.049 (± 0.205)	30	38
25ºC	MP only	6	22.786 (± 0.230)	18	18.309 (± 0.180)	18	16.589 (± 0.239)	20	27
	MP + AuNP	6	18.399 (± 0.623)	29	18.309 (± 0.309)	27	9.983 (± 0.341)	20	46

3.4.3. Bioconcentration of gold by P. microps

The actual concentrations of gold in freshly prepared (0h) and 48h old test media determined by atomic absorption spectroscopy in the test beakers containing fish are indicated in Table 12. The mean of all the determinations made in fresh media was much lower than the nominal concentration of AuNP (0.2 $mg.L^{-1}$) and than the actual concentrations estimated from the absorbance using the model of Figure 2. This may be at least in part because of the heterogeneous distribution of AuNP in test media and thus sampling bias. Therefore, for future bioassays, the use of the whole test volume instead of a sample of it is recommended. Based on chemical determinations, no significant decay of gold in test media occur. However, because of the potential bias introduced during sampling and by the low gold concentrations tested, this result should be considered with caution.

Table 12. Actual concentration of gold (Au) on the fresh (0h) and old (48H) media determined by atomic absorption spectroscopy. Conc. – concentration; T - Temperature; Treat. – Treatment; Dev. - Deviation; N - number of samples analyzed.

			Fresh Media		Old Media		
T(ºC)	Treat.	Nominal Conc. (mg/L)	Actual Conc. (mg/L)	%Dev.	N	Actual Conc. (mg/L)	%Decay
20ºC	AuNP	0.200	0.061	70	12	0.072 (±0.004)	0
	AuNP + MP	0.200	0.053	74	12	0.075 (±0.006)	0
25ºC	AuNP	0.200	0.060	61	12	0.058 (±0.003)	3
	AuNP + MP	0.200	0.052	74	12	0.058 (±0.001)	0
Overall mean		0.200	0.056 (± 0.002)	72	46	0.066	0

The concentration of gold determined by chemical analysis in the whole body of fish after 96h of exposure to AuNP, alone or in combination with MP is shown in Table 13. All the fish had gold in their body, indicating that they have taken up the metal from the test media.

Table 13. Concentration of gold in *P.microps* whole body after 96h of exposure to 5nm AuNP in the presence and absence of MP at 20ºC and 25ºC. Results are expressed as mean and standard error of the mean. LQ – Limit of quantification.

Temperature	Treatment	N	Au / µg. g^{-1}
20ºC	Control	6	<LQ
	AuNP	6	0.24 ± 0.09
	AuNP + MP	6	0.13 ± 0.02
25ºC	Control	6	<LQ
	AuNP	6	1.07 ± 0.78
	AuNP + MP	5	0.55 ± 0.25

Fish exposed to 25ºC had more gold in their body than those exposed to 20ºC, and albeit no statistically significant differences were found between the treatments and the two temperatures ($F_{2,29}$ = 0.756, p > 0.05), this indicates that temperature rise may increases the uptake of gold, reduces its excretion and/or changes other biological processes. The decay of AuNP in old test media where the fish were maintained for 48h, determined by spectrophotometry, was higher at 25ºC than at 20ºC (Table 8), supporting an increased uptake of gold by fish at the highest temperature and excluding an increased gold excretion. At higher temperatures and in general, the metabolism of fish is accelerated (Clarke & Johnston, 1999). Thus, at 25ºC P. microps is expected to increase its metabolism and filter more water to get oxygen enough to respond the increased energy demands, a process that could have lead to a higher uptake of gold from the water.

Fish exposed to AuNP alone had more gold in their tissues (≈ 2 folds) than those exposed simultaneously to AuNP and MP. These results indicate that MP influence the uptake and/or excretion of the metal by fish. At least two not mutually exclusive hypothesis may be raised to explain a decreased uptake of gold by fish: (i) binding of AuNP or other gold form present in test media to MP with the resulting complex being not (or being less) up taken by fish; and (ii) somehow MP interfere directly with the biological process(es) of gold uptake by fish. Results of previous sections indicated that at 25ºC in the absence of fish the AuNP decay was lower in the presence of MP than when they were not present (Table 7); moreover, at both temperatures, the actual concentrations of MP in fresh media (Table 9) were lower in the presence of AuNP than when MP were the only substance present. However, because at 20ºC and in the absence of fish, the

presence of MP did not significantly changed the AuNP decay, other processes seem to have contributed to the reduced gold body burden in the presence of MP. In beakers containing fish (Table 8), the maximal increase of AuNP decay in the presence of MP was of 19%, while in the absence of fish (Table 7) the MP didn't exacerbate the decay of AuNP; in addition, in test beakers without fish the AuNP decay in the absence and presence of MP was almost similar at 20ºC but different at 25ºC (Table 7), while the opposite was found when the fish were present (Table 8). These evidences indicate that fish removed the test substances from the test media (up taken them), and that MP influence the biological processes responsible for AuNP uptake, and likely other biological processes related with gold-induced stress inside the organism, and vice-versa as evident from (Table 11).

The bioconcentration factors (BCF) calculated from the gold concentrations in 48h old media and in the fish body determined by chemical analysis (BCF-A) are indicated in (Table 14). The values ranged from 1.7 determined for fish exposed at 20ºC in the presence of MP to 18.5 in fish exposed to 25ºC in the absence of MP. Because the potential aggregation phenomena that may have introduced bias in the sampling and thus a underestimation of the real content of gold present in test media, the BCF were also calculated from the AuNP spectrophotometric determinations (BCF-B). By this approach, BCF-B of 0.69 and 1.1 were determined for fish exposed to 20ºC, indicating no accumulation of the metal. At 25ºC, the BCF-B was 5.1 and 3.7, indicating that fish bioconcentrated the gold present in the water. Therefore, independently of the potential methodological problems, the results of Table 14 indicate that *P. microps* juveniles exposed to AuNP through the water are able to bioconcentrate gold in its body, and that the process is temperature dependent and the presence of MP may interfere with the process. This is a concerning finding because *P. microps* is an important prey for higher predators during all the phases of its life cycle, including to species of human consumption. Therefore, its capability to bioconcentrate gold from water suspended AuNP increases the risk of exposure and effects to top predators and humans, especially at 25ºC, an ecological relevant Spring/summer water temperature in natural habitats of South Europe, that is expected to become also common in several other regions where *P. microps* occur.

Table 14. Bioconcentration factors (BCF-A and BCF-B) calculated from the gold concentrations in test media (ASW) determined by chemical analysis (A) and by spectrophotometry (B) respectively. BCF = concentration of gold in the fish body / concentration of gold in ASW. Temp. – temperature; Treat – treatment; Conc. – concentration

T (ºC)	Treat.	Conc. gold in ASW (A) (ppm)	Conc. gold in ASW (B) (ppm)	Conc. gold in fish (ppm)	BCF-A	BCF-B
20	AuNP	0.072	0.226	0.24	3.3	1.1
	AuNP+MP	0.075	0.188	0.13	1.7	0.69
25	AuNP	0.058	0.211	1.07	18.5	5.1
	AuNP+MP	0.058	0.150	0.55	9.5	3.7

3.4.4. Water and fish morphometric parameters

During each 48h interval (time renewal interval), the variation of water temperature, pH, and salinity in each test beaker was always lower than 1ºC, 0.1 pH units, and 1 $g.L^{-1}$, respectively, and the oxygen levels were always higher than 8 $mg.L^{-1}$ (Table A 1). No mortality was recorded in the control groups. Therefore, the bioassay can be considered valid according the OECD guidelines for acute testing with juvenile fish (OECD, 1992) relatively to these parameters. Additionally, no significant differences in the fish total length (20ºC: $F_{3,48} = 1.835$, $p > 0.05$; 25ºC: $F_{3,48} = 0.793$, $p > 0.05$) and weight (20ºC: $F_{3,48} = 0.475$, $p > 0.05$; 25ºC: $F_{3,48} = 0.022$, $p > 0.05$) were found among treatments. Additionally, no differences were found between the means of the length ($t_{102} = 1.941$, $p > 0.05$) and weight ($t_{102} = 1.209$, $p > 0.05$) of the fish exposed at each temperature.

3.4.5. Effects of AuNP and MP on fish predatory performance and biomarkers

The effects of AuNP on the predatory performance of fish assessed through the number of ingested *Artemia* nauplii are shown in Figure 5 and in Table 15. Significant differences in the performance of fish exposed to different treatments indicating adverse effects of the tested substances on the fish predatory performance; no significant differences between fish exposed to distinct temperatures were found indicating that temperature rise from 20ºC to 25ºC did not significantly affect the fish predatory performance; and no significant interaction was found suggesting that in the range of

concentrations tested, temperature did not interact with the effects of the substances on the predatory performance of fish (Table 15). Considering the overall means per treatment (Table 15), fish exposed to AuNP either in the presence or in the absence of MP showed a significant decrease of the predatory performance relatively to fish from the control group and those exposed to MP alone which are not significantly different among them. The comparison of fish exposed to different treatments per temperature, separately, (Figure 5) indicated significant differences at both 20ºC (one-way ANOVA: $F_{3,50}$=4.911, $p<0.05$) and 25ºC (one-way ANOVA: $F_{3.48}$=3.370, $p<0.05$). At 20ºC, both treatments with AuNP (i.e. AuNP alone and AuNP in combination with MP) caused significant reduction of the percentage of ingested *Artemia*, whereas at 25ºC only the treatment with AuNP and MP caused a significant reduction of predatory performance (Figure 5). Thus, AuNP decreased the predatory performance of the fish, an effect that may cause the reduction of the individual fitness due to growth delay, reduction of the health condition, and decrease of the reproductive output due to the difficulty of capturing prey. These effects can lead to a decrease of population fitness caused by increased mortality and generation time (due to growth delay), and decrease of the reproduction rate. Behavioural impairment caused by other metallic nanoparticles (titanium dioxide and copper) has been reported (Chen, et al., 2011; Sovová, et al., 2014) and exposure to 5, 15 and 45 nm AuNP have decreased the burrowing kinetics of *Scrobicularia plana* (Pan, et al., 2012) however, to our knowledge, the effects of AuNP on predatory performance wasn't investigated in previous studies.

In the range of concentrations and temperatures tested, AuNP and MP, either alone or combined, and temperature had no significant effects on *P. microps* acetylcholinesterase activity (Figure 6; Table 15). The interaction between the two factors (treatment and temperature) was also not significant. Therefore, the tested substances and temperature change did not disrupt the cholinergic function at least through AChE inhibition. Contrary to these results, Pan *et al.* (2012) detected increased AChE levels on *Scrobicularia plana* caused by exposure to AuNP of 15nm and 40nm. Additionally, in a previous study with *P. microps* juveniles a significant decrease of AChE activity (29.3%) was found under exposure to 0.184 $mg.L^{-1}$ of the same MP at 20ºC (Oliveira, et al., 2013). This effect wasn't found on this work probably because Oliveira *et al.* used smaller fish (1.0 to 1.2 cm of length) which are probably more sensitive.

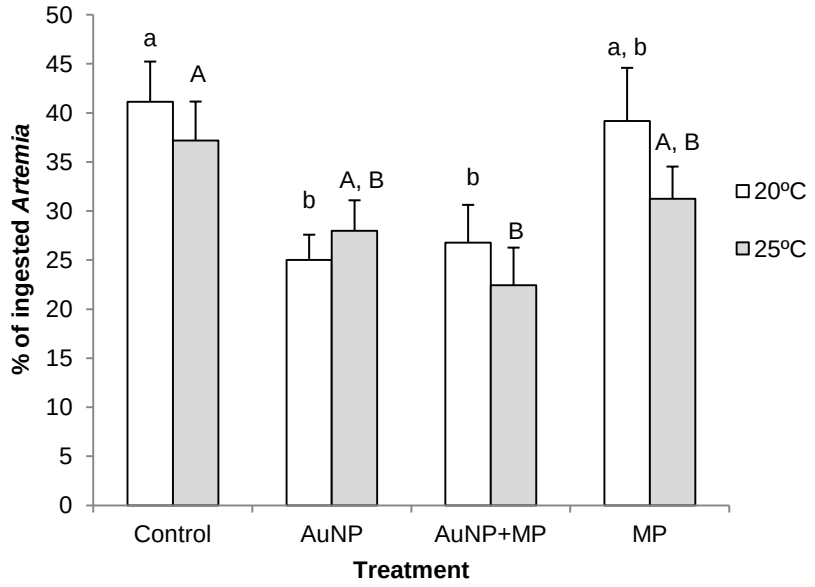

Figure 5. Predatory performance of *Pomatoschistus microps*, assessed through the percentage of Artemia naupli, after 96h of exposure to 5nm gold nanoparticles (AuNP) and to microplastics (MP), alone and in combination at 20ºC and 25ºC. Black and grey bars express the results obtained at 20ºC and 25ºC respectively. Results are expressed as percentage means of fish performance with corresponding standard error bars (n = 15). MP – fish exposed to MP alone (0.184mg.L^{-1}). AuNP – fish exposed to AuNP alone (0.2 mg.L^{-1}). AuNP+MP – fish exposed simultaneously to AuNP (0.2 mg.L^{-1}) and MP (0.184mg.L^{-1}). Different letters above the bars indicate statistically significant differences (1-way ANOVA and Tukey's multi-comparison test).

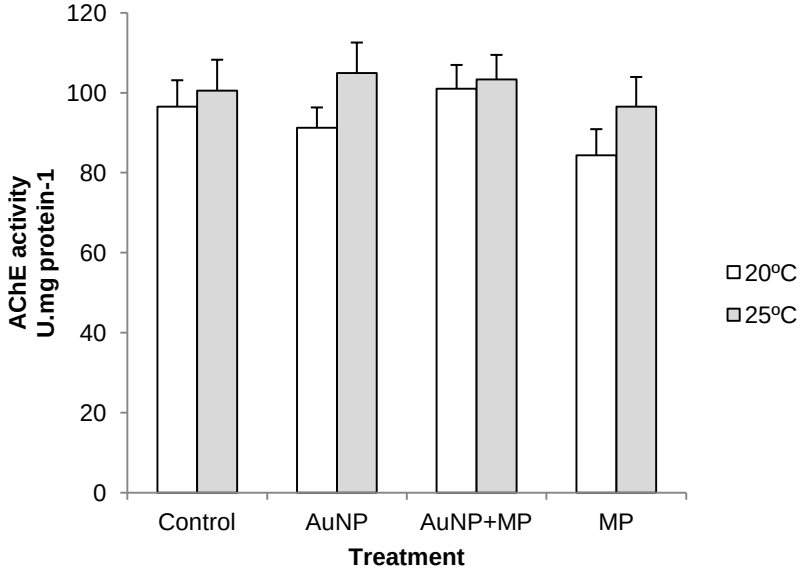

Figure 6. Acetylcholinesterase activity on head homogenates of *Pomatoschistus microps* after 96h of exposure to 5nm gold nanoparticles (AuNP) and to microplastics (MP), alone and in combination at 20ºC and 25ºC. Black and grey bars express the results obtained at 20ºC and 25ºC respectively. Results are expressed as means ± standard errors (n = 9). MP – fish exposed to MP alone (0.184mg.L^{-1}). AuNP – fish exposed to AuNP alone (0.2 mg.L^{-1}). AuNP+MP – fish exposed simultaneously to AuNP (0.2 mg.L^{-1}) and MP (0.184mg.L^{-1}). U - nmol.min^{-1}.

Table 15. Summary of the statistical results obtained with the two-way ANOVA (α = 0.05) to compare individual parameters of fish exposed to different treatments, to different temperatures, and the interaction between the two factors. Significant differences between each treatment were assessed by the post hoc Tukey's multi-comparison test when significant differences between treatments were detected by the two-way ANOVA. When applied, different letters indicate statistically significant differences. *df* – degrees of freedom; Sig. – Significant; Pred. Perf. – Predatory performance; AChE – activity of acetylcholinesterase; GST- activity of glutathione s-transferases; EROD – activity of ethoxyresorufin-O-deethylase; LPO – lipid peroxidation levels; SEM – standard error of the mean

Endpoint	Factor	Mean ± SEM	Sig. differences	*df*	F	p
Pred. Perf.						
	Treatment			3, 98	7.559	0.000
	Control	39.14% ± 2.8	a			
	AuNP	26.49% ± 2.0	b			
	AuNP+MP	24.61% ± 2.7	b			
	MP	35.21% ± 3.0	a			
	Temperature			1, 98	1.684	0.197
	20ºC	33.02% ± 1.9				
	25ºC	29.71% ± 1.8				
	Interaction			3, 98	0.733	0.535
AChE						
	Treatment			3, 64	1.088	0.361
	Control	98.53 U ± 5.0				
	AuNP	98.10 U ± 4.7				
	AuNP+MP	102.19 U ± 4.1				
	MP	90.44 U ± 5.0				
	Temperature			1, 64	2.888	0.094
	20ºC	94.34 U ± 3.3				
	25ºC	100.83 U ± 3.8				
	Interaction			3, 64	0.366	0.778
GST						
	Treatment			3, 64	1.540	0.213
	Control	29.26 U ± 2.8				
	AuNP	35.64 U ± 1.9				
	AuNP+MP	33.53 U ± 1.7				
	MP	32.73 U ± 3.1				
	Temperature			1, 64	7.720	0.007
	20ºC	35.76 U ± 1.8	*			
	25ºC	29.82 U ± 1.4	*			
	Interaction			3, 64	2.879	0.043

Table 15. cont.

Endpoint	Factor	Mean ± SEM	Sig. differences	*df*	F	Sig.
EROD						
	Treatment			3, 17	0.394	0.759
	Control	0.14 U ± 0.02				
	AuNP	0.22 U ± 0.04				
	AuNP+MP	0.24 U ± 0.02				
	MP	0.35 U ± 0.13				
	Temperature			1, 17	3.451	0.081
	20ºC	0.12 U ± 0.02				
	25ºC	0.35U ± 0.06				
	Interaction			3, 17	0.649	0.594
LPO						
	Treatment			3, 64	5.369	0.002
	Control	0.56 U ± 0.07	a, b			
	AuNP	0.44 U ± 0.04	a			
	AuNP+MP	0.72 U ± 0.08	b			
	MP	0.71 U ± 0.08	b			
	Temperature			1, 64	0.845	0.362
	20ºC	0.56U ± 0.04				
	25ºC	0.65U ± 0.06				
	Interaction			3, 64	0.553	0.648

Regarding GST, there were no significant differences among treatments (Table 15) but a significant effect of temperature, and a significant interaction were found. Considering the overall means, at 25ºC, the GST activity of fish was significantly lower than at 20ºC. The significant interaction suggests that temperature influenced the effects of the both particles on GST activity. The comparison of GST activity of fish exposed to different temperatures per treatment (Figure 7; Table 16), indicates significant lower GST activity at 25ºC in fish exposed to AuNP alone and in fish exposed to MP alone than at 20ºC. Although these results deserve some caution in their interpretation because no significant differences among treatments were found considering the overall means (Table 15) and the means of different treatments compared per temperature separately (one-way ANOVA; 20ºC: $F_{2,32}$=2.407, p>0.05; 25ºC: $F_{2,32}$=1.900, p>0.05), they suggest an influence of temperature on the effects of AuNP alone and MP alone (but not in combination) of GST activity in good agreement with the significant interaction between the two factors (treatment and temperature) indicated by the two-way ANOVA (Table 15). This may be of high concern to the fish fitness in their natural environment due to the crucial role that GST has on the detoxification process of xenobiotics and on the protection against oxidative stress. Although no effects upon GST caused by exposure of *P. mirops* to AuNP were detected in the present work, in a previous study, AuNP (5; 15 and 40 nm) induced

the GST of marine bivalve *Scorbicularia plana* (Pan, et al., 2012). On an *in vitro* study with Balb/3T3 mouse fibroblast cells, GST was also found to be up regulated when cells were exposed to 15 nm AuNP and down regulated in cells treated with 5nm AuNP (Gioria, et al., 2014). In agreement with our results Oliveira et al. (2013) didn't found any effects upon GST caused by exposure of *P. microps* to 0.184 mg.L^{-1} of MP.

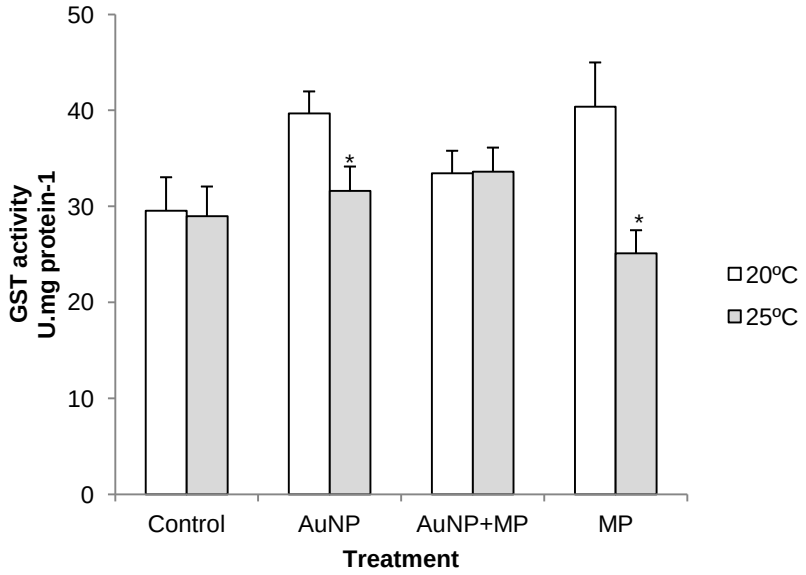

Figure 7. Glutathione S-Transferase activity of *Pomatoschistus microps* after 96h of exposure to 5nm gold nanoparticles (AuNP) and to microplastics (MP), alone and in combination at 20ºC and 25ºC. Black and grey bars express the results obtained at 20ºC and 25ºC respectively. Results are expressed as means ± standard errors (n = 9). * - Significantly different from the same treatment made at different temperature (Student's *t*-test p<0.05). MP – fish exposed to MP alone (0.184 mg.L^{-1}). AuNP – fish exposed to AuNP alone (0.2 mg.L^{-1}). AuNP+MP – fish exposed simultaneously to AuNP (0.2 mg.L^{-1}) and MP (0.184mg.L^{-1}). U - nmol.min^{-1}.

Table 16. Results of the student's *t*-test conducted to examine the effects of temperature on the GST activity on each treatment. For that, the means obtained in each treatment at 20ºC and 25ºC were compared doing an independent samples t-test (α=0.05). U - nmol.min^{-1}. *df* – degrees of freedom; Sig. – level of significance.

Independent Samples *t*-test			
Treatment	***df***	***t* value**	**Sig.**
Control at 20ºC vs. Control at 25ºC	16	0.123	0.904
AuNP at 20ºC vs. AuNP at 25ºC	16	2.339	0.033
AuNP+ MP at 20ºC vs. AuNP + MP at 25ºC	16	0.049	0.961
MP at 20ºC vs MP at 25ºC	16	2.920	0.010

AuNP and MP, either alone or in combination, didn't induced significant effects upon EROD activity (Figure 8; Table 15); no significant differences on the EROD activity of fish exposed to different temperatures, and no significant interaction between temperature and treatments were found. These results may be due to the high variability in EROD determinations which may be due to the low enzymatic content of the fractions used. It would have been more appropriate to perform such determinations in isolated fish liver. However, *P. microps* juveniles of the size used in the present study have a very small liver and thus a considerable higher number of fish would have been needed to prepare pools of livers for EROD activity determinations and thus, a pool of whole body (without the head) fraction was used instead which had a very low enzymatic activity also increasing the error and variability of the analysis. Thus, the interpretation of the results should be careful. In control fish, where a small variability was found, the enzymatic levels are similar at the two temperatures, suggesting that the increase of 5ºC in temperature had no effects on *P. microps* EROD activity. Although not significant, at 25ºC, fish exposed to AuNP alone and to MP alone had higher EROD activity than those of the control group, suggesting that both chemicals (but not their combined effect) are able to induce the activity of this enzyme at high temperatures. Thus, our results cannot exclude the involvement of EROD activity of the biotransformation of AuNP and MP, and more investigation on this topic is needed.

The results of LPO levels are presented in Figure 9. Significant differences among treatments (Table 15) but not between temperatures, and no significant interaction were found. Considering the overall means, no significant differences in LPO levels between fish exposed to treatments containing AuNP, MP either alone or in combination, and fish from the control group were found. However, fish exposed to treatments containing MP (either alone or in combination with AuNP) had higher LPO levels than those exposed to AuNP alone. The comparison of the LPO levels separated per temperature (Figure 9) showed significant differences among fish exposed to different treatments at 20ºC but not at 25ºC ($F_{3,32}$=5.715, $p<0.05$; $F_{3,32}$=1.743; $p>0.05$). At 20ºC, LPO levels of fish exposed to AuNP alone were not significantly different from those of the control group, indicating that at this temperature, the nanoparticles tested did not induced lipid peroxidation damage on the fish. On the contrary, MP alone and in combination with AuNP significantly increased the LPO levels of fish indicating that they induced lipid peroxidation. Although no significant differences among treatments were found at 25ºC, probably because of the relatively high variability within each treatment, the results suggest interesting evidences. First, in the control groups, higher levels of LPO were found in fish exposed at 25ºC than

at 20ºC; Second, at 25ºC, fish exposed to AuNP alone had lower LPO levels than those of the control group (Figure 9), suggesting that AuNP may have a protective effect against oxidative stress which is in good agreement with the antioxidant properties of gold nanoparticles previously suggested: chitosan stabilized AuNP were found to interact and capture hydroxyl radicals (Yakimovich, et al., 2008) decreasing the concentration of these radicals in solution, these observations were found to be size and surface type and concentration dependent; in other study it was detected that AuNP inhibit lipid peroxidation *in vitro* at a rate up to 47% (Prakash, et al., 2013); in other work using chitosan stabilized AuNP it was also found that AuNP exhibit antioxidant properties since they were able to scavenge the free radical 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS•), the smaller nanoparticles showed the greater antioxidant activity (Duy, et al., 2013). However, because in studies with other species, including the rainbow trout (*Oncorhynchus mykiss*) and the blue mussel (*Mytilus edulis*) an increase of ROS production (Farkas, et al., 2010) and oxidative damage (Tedesco, et al., 2010) were respectively found, the effects may be species specific and is not certain that the antioxidant effects referred above manifest *in vivo* so more research is needed to clarify this particular subject; Third, when fish are exposed simultaneously to AuNP and MP, the LPO levels are higher than under exposure to AuNP alone suggesting that the possible AuNP protective role against oxidative damage was not enough to protect from the oxidative damage induced by MP and/or temperature. Finally, because the LPO levels are similar under MP exposure to 20 and 25ºC, and higher than those of the control group (especially at 20ºC), MP caused oxidative stress and damage in *P. microps*, as well as temperature, but temperature does not significantly influence the MP induced oxidative damage at least in this range of chemical and thermally induced stress.

The increase of fish LPO levels with the temperature rise is in agreement with findings from studies in the literature, indicating that high temperatures cause oxidative stress and lipid peroxidation damage. For example, in a study with sea bass (*Dicentrarchus labrax*) higher LPO levels were found in fish exposed to 25ºC comparatively to fish exposed at 18ºC (Almeida, et al., 2014). Other study also detected higher LPO levels in response to thermal stress induced by increasing the temperature by 1ºC/h in thin-lipped Mullet (*Liza ramada*), Sargo (*Diplodus sargus*) and in sea Bass (*Dicentrarchus labrax*) (Vinagre, et al., 2012). Accordingly, in thorn fish (*Terapon jarbua*) exposed at 28, 32 and 36ºC the measured LPO levels increased with water temperature (Chien & Hwang, 2001).

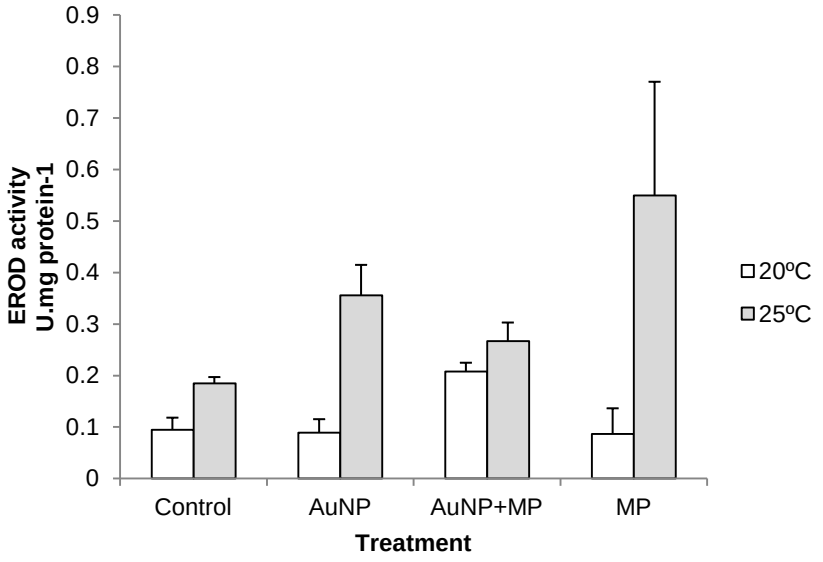

Figure 8. Ethoxyresorufin-O-deethylase activity of *Pomatoschistus microps* after 96h of exposure to 5nm gold nanoparticles (AuNP) and to microplastics (MP), alone and in combination at 20ºC and 25ºC. Results are expressed as means ± standard errors (n = 3). MP – fish exposed to MP alone (0.184mg.L^{-1}). AuNP – fish exposed to AuNP alone (0.2 mg.L^{-1}). AuNP+MP – fish exposed simultaneously to AuNP (0.2 mg.L^{-1}) and MP (0.184mg.L^{-1}). U - nmol.min^{-1}.

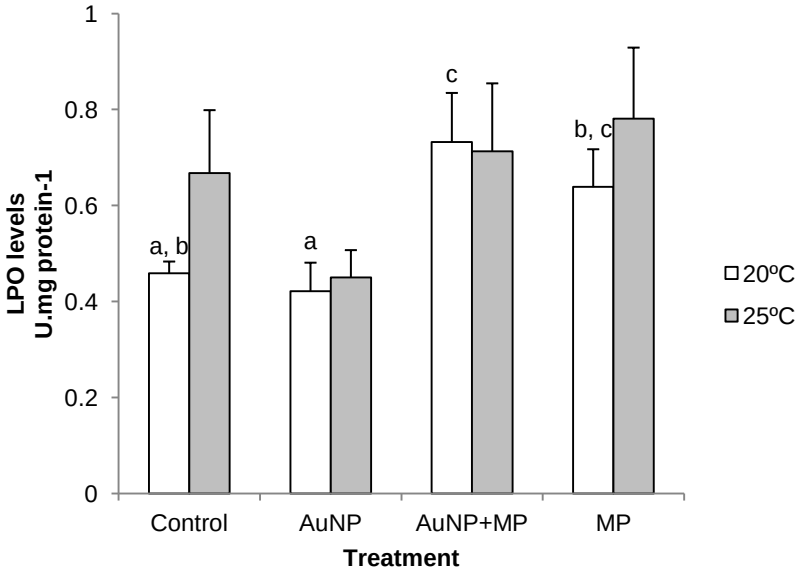

Figure 9. Lipid Peroxidation levels on body homogenates of *Pomatoschistus microps* after 96h of exposure to 5nm gold nanoparticles (AuNP) and to microplastics (MP), alone and in combination at 20ºC and 25ºC. Black and grey bars express the results obtained for 20ºC and 25ºC bioassays respectively. Results are expressed as means ± standard errors (n = 9). MP – fish exposed to MP alone (0.184mg.L^{-1}). AuNP – fish exposed to AuNP alone (0.2 mg.L^{-1}). AuNP+MP – fish exposed simultaneously to AuNP (0.2 mg.L^{-1}) and MP (0.184mg.L^{-1}). Different letters above the bars indicate statistically significant differences (1-way ANOVA and Tukey's multi-comparison test). U – nmol of TBARS.min^{-1}.

4. Conclusions

The methods used to estimate the size and shape of 5 nm AuNP from their UV-Vis spectra, adapted from Haiss et al. (2007) and Amendola & Meneghetti (2009), respectively, and validated in the present study for use in saltwater are cost-effective methods. However, they may lose precision for smaller particles, as previously discussed by the authors for other suspension media, and at low AuNP concentrations like the ones used on toxicological assays. The sensitivity and accuracy of the equipment used are also very important. The conclusions regarding the spectrofluorimetry method adapted here to determine the concentrations of MP in saltwater are similar to those indicated for the method used for AuNP basic characterization.

In this study, it was observed that the spectrophotometric properties of AuNP suspensions prepared in ASW (18 $g.L^{-1}$) and u.p. water were different, indicating that suspension media influences the properties of these nanoparticles. In ASW, AuNP properties changed over time indicating that in saltwater AuNP with 5nm of diameter are not stable and may form aggregates and precipitate which make difficult to assess their toxicity, especially relatively to specific size and shape. However, allowing these changes to occur may mimic what happens in real scenarios, such as estuaries and other coastal areas where *P. microps* lives, where the conditions are not constant. MP were also found to decay in test media, likely due to aggregation and precipitation. Moreover, our results suggest that AuNP interact with MP in test media

Fish exposed to AuNP (5 nm) for a short period of time (96h) absorbed gold from the water reaching concentrations of 0.24 $\mu g.g^{-1}$ at 20ºC and 1.07 $\mu g.g^{-1}$ at 25ºC confirming our first hypotheses (fish exposed to AuNP through the water uptake gold). Because *P. microps* is an important prey for higher predators, including edible species to humans, these findings raise a high concern regarding the increased risk of exposure and induction of adverse effects on higher predators and humans consuming them regularly. Moreover, due to global warming, water temperatures of 25ºC that induced a higher uptake of gold by fish in the present study, are expected to be more frequent, especially in ecosystems of South Europe, including several impact estuaries and other coastal areas inhabited by this species where fisheries are important economic activities. At high temperatures, the accumulation of several common pollutants is also higher (Segner, et al., 2014), increasing the risks of exposure to top predators and humans through the consumption of contaminated species.

According to the results obtained, 5 nm of diameter AuNP significantly decreased the predatory performance of *P. microps* juveniles, corroborating our second hypotheses

(AuNP (5 nm diameter) are toxic to *P. microps* juveniles at exposure concentrations in the ppb range). In the wild, the reduction of the ability of fish to capture prey is likely to result in growth delay, decreased health status and reproductive output, ultimately leading to death. The increase of mortality rate in the population and of generation time (due to growth delay), and/or the reduction of the reproduction rate is expected to decrease the population fitness and thus its ecological function. Since *P. microps* populations are most important to control the zooplankton community during larval and early juvenile phases (avoiding its over developement and the depletion of primary producers populations (phytoplankton) potentially resulting from the zooplankton increase), and are an important prey for several higher predators during the entire life cycle (Quintaneiro, et al., 2008), adverse effects of *P. microps* populations may reduce ecosystem biodiversity and functioning and its services. No significant effects of AuNP on AChE activity were found indicating that these nanoparticles do not affect the cholinergic function (at least through AChE inhibition) of *P. microps*, an effect that may be of interest also in relation to human health. Relatively to the respective control groups, no significant effects in GST activity were found in fish exposed to AuNP at 20ºC and 25ºC, suggesting that this enzyme is not involved in the biotransformation of AuNP. However, because a significant reduction of GST activity at 25ºC relatively to the corresponding response at 20ºC was found in fish exposed to AuNP, the role of temperature is not clear. No lipid peroxidation caused by AuNP was found. Regarding EROD, because the high variability in the analysis made, our results cannot exclude the involvement of EROD in the biotransformation of AuNP by *P. microps* at least at 25ºC.

MP alone caused an increase of LPO levels indicating their ability to induce oxidative stress resulting in lipid peroxidation damage, with no significant effects in any of the other parameters analyzed, despite an 8% reduction of AChE, an effect of MP previously reported for this species (Oliveira, et al., 2013). When fish were simultaneously exposed to the two tested chemicals, AuNP seem to have had some protective action against MP-induced LPO. Thus, in addition to interactions between MP and AuNP in test media, there were toxicological interactions between the chemicals inside the fish (as also suggested by the studies based on the concentrations of AuNP and MP in test media). Thus, toxicological interactions occur when fish are simultaneously exposed to AuNP and MP, a conclusion that corroborates our third hypothesis (MP (polyethylene 1-5 µm spheres) interact with the effects of AuNP on *P. microps* juveniles).

As previously indicated, the rise of temperature by 5ºC (20ºC to 25ºC) increased the levels of gold on fish body. In addition, the thermally-induced stress increased the lipid

peroxidation damage in control fish as indicated by the higher LPO levels in the control group at 25ºC relatively to the control group at 20ºC. Moreover, temperature may influence the GST and EROD activity responses to stress induced by AuPN alone and MP alone, respectively. In the treatments with AuNP alone and MP alone at 25ºC, a significant reduction of GST levels relatively to the corresponding treatments at 20ºC, suggesting an influence of temperature on the individual effects of these chemicals, as also suggested by the significant interaction detected by the two-way ANOVA. Therefore, the above summarized findings, indicate an influence of temperature rise from 20ºC to 25ºC on the toxic effects of chemicals, supporting our fourth hypothesis (the rise of temperature from 20ºC to 25ºC influences the effects of chemicals on *P. microps* juveniles).

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6. Annex

Table A 1. Water physicochemical parameters registered during the multi-stressors bioassay. The results are expressed as means ± standard deviation. T – Temperature at which the assay was carried out; D.O. – Dissolved Oxygen; Temp. – Registered water temperature.

T (ºC)	Time	pH	D.O (mg.L^{-1})	Temp. (ºC)	Salinity (g.L^{-1})
20ºC	0h	8.55 ± 0.13	8.66 ± 0.13	19.91 ± 0.52	18 ± 0
	24h	8.56 ± 0.18	8.94 ± 0.24	19.65 ± 0.65	18.57 ± 0.68
	48h	8.56 ± 0.15	9.11 ± 0.23	19.31 ± 0.57	18.63 ± 0.61
25ºC	0h	8.59 ± 0.08	8.36 ± 0.06	24.44 ± 0.37	18 ± 0
	24h	8.69 ± 0.05	8.14 ± 0.13	25.24 ± 0.74	18.17 ± 0.59
	48h	8.69 ± 0.04	8.14 ± 0.11	25.16 ± 0.57	18.30 ± 0.52